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(54) Title: KINASE RECEPTOR ACTIVATION ASSAY															
(57) Abstract															
<p>An assay for measuring activation (i.e., autophosphorylation) of a tyrosine kinase receptor of interest is disclosed. a) A first solid phase is coated with a substantially homogeneous population of cells so that the cells adhere to the first solid phase. The cells have either an endogenous tyrosine kinase receptor or have been transformed with DNA encoding a receptor or "receptor construct" and the DNA has been expressed so that the receptor or receptor construct is presented in the cell membranes of the cells. b) A ligand is then added to the solid phase having the adhering cells, such that the tyrosine kinase receptor is exposed to the ligand. c) Following exposure to the ligand, the adherent cells are solubilized, thereby releasing cell lysate. d) A second solid phase is coated with a capture agent which binds specifically to the tyrosine kinase receptor, or, in the case of a receptor construct, to the flag polypeptide. e) The cell lysate obtained in step c) is added to the wells containing the adhering capture agent so as to capture the receptor or receptor construct to the wells. f) A washing step is then carried out, so as to remove unbound cell lysate, leaving the captured receptor or receptor construct. g) The captured receptor or receptor construct is exposed to a labelled anti-phosphotyrosine antibody which identifies phosphorylated residues in the tyrosine kinase receptor. h) Binding of the anti-phosphotyrosine antibody to the captured receptor or receptor construct is measured.</p>															

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KINASE RECEPTOR ACTIVATION ASSAYBACKGROUND OF THE INVENTIONField of the Invention

This invention relates to a kinase receptor activation (KIRA) assay.

- 5 In particular, the invention relates to an assay for measuring autophosphorylation of the kinase domain of a receptor protein tyrosine kinase (rPTK) using a kinase receptor activation, enzyme-linked immunosorbent assay (KIRA ELISA).

Description of Related Art

- 10 One mechanism for signal transduction in animals involves protein phosphorylation. Protein phosphorylation involves the action of protein kinase, an enzyme that transfers a phosphate group from a phosphate donor onto an acceptor amino acid in a substrate protein. Protein phosphatases provide a means for reversing the signal when the stimulus is removed.

- 15 Protein kinases have multiple substrates, and classification of the protein kinases is based on the acceptor amino acid specificity. The two most well characterized protein kinases are the protein kinases with a protein alcohol group as acceptor called protein serine/threonine kinases and the protein kinases with a protein phenolic group as acceptor called
20 protein tyrosine kinases (Hunter, Methods in Enzymology 200:3-9 [1991]).

- The most well known type of signal-transducing protein kinases are growth factor receptor protein tyrosine kinases (rPTKs). rPTKs usually comprise a large, glycosylated, extracellular ligand binding domain (ECD) and an intracellular domain (ICD) which contains a tyrosine kinase
25 catalytic domain. A single hydrophobic transmembrane (TM) domain connects the ECD and ICD. Examples of rPTKs include the insulin receptor, epidermal growth factor receptor (EGF-R), platelet-derived growth factor receptor (PDGF-R), insulin-like growth factor 1 receptor (IGF-1-R), and the HER2 receptor, to name a few. See, for example, Ullrich and Schlessinger Cell
30 61:203-212 (1990) and Fantl et al., Annu. Rev. Biochem. 62:453-481 (1993). rPTKs can phosphorylate exogenous protein substrates and intrinsic tyrosine residues via their catalytic tyrosine kinase domain. The intrinsic tyrosine residues normally reside in the ICD of the rPTK (see Figure 1 herein). Activation of the intracellular kinase domain of rPTKs appears
35 to be mediated by receptor oligomerization which results from the conformational alteration of the ECD upon ligand binding thereto. See

Ullrich and Schlessinger, *supra*.

Serine-threonine kinases have also been disclosed in the literature. While most of the known protein serine-threonine kinases are cytoplasmic proteins, a family of mammalian transmembrane receptors with serine-threonine kinase domains has recently been found. Members of this receptor family have been described as binding TGF- β 's and activin. For reviews of serine-threonine kinases, see Sale, G., Biochem. Soc. Transactions 20: 664-670 (1992); ten Dijke et al., Prog. in Growth Factor Res. 5: 55-72 (1994); and Mathews, L., Endoc. Rev. 15(3): 310-325 (1994).

Various assays have been developed which measure tyrosine kinase activity. Some of these assays measure the ability of a tyrosine kinase enzyme to phosphorylate a synthetic substrate polypeptide. For example, an assay has been developed which measures growth factor-stimulated tyrosine kinase activity by measuring the ability of the kinase to catalyze the transfer of the γ -phosphate of ATP to a suitable acceptor substrate. See Pike, L., Methods of Enzymology 146:353-362 (1987) and Hunter, Journal of Biological Chemistry 257(9):4843-4848 (1982), for example. In this assay, the use of [γ - 32 P]ATP permits the radioactive labeling of the phosphorylated substrate, which is a synthetic tyrosine-containing peptide. Others have described protein kinase assays wherein incorporation of 32 P into a tyrosine kinase receptor, such as the EGF receptor (see Donato et al., Cell Growth Differ. 3:259-268 [1992]), insulin receptor (see Kasuga et al., Journal of Biological Chemistry 257(17):9891-9884 [1982] and Kasuga et al., Methods in Enzymology 109:609-621 [1985]), and liver growth hormone receptor (see Wang et al., Journal of Biological Chemistry 267(24):17390-17396 [1992]), is measured.

The discovery of anti-phosphotyrosine antibodies has provided a non-radioactive, alternative means for measuring phosphorylation of tyrosine residues. For example, White and Backer (Methods in Enzymology 201:65-67 [1991]) mention polyclonal antibodies which selectively bind to phosphotyrosine and are considered to be useful for studying rPTKs. An anti-phosphotyrosine monoclonal antibody was used in one of the assays referred to in Madden et al. (Anal. Biochem. 199:210-215 [1991]), which measured phosphatase activity toward the insulin receptor. Anti-phosphotyrosine antibodies were also used by Cleaveland et al., in their protein tyrosine kinase ELISA assay. See Cleaveland et al., Analytical Biochemistry 190:249-253 (1990). The method of Cleaveland et al. utilizes purified high-activity oncogene tyrosine kinases, v-src and v-fps, and

measures the ability of these tyrosine kinases to phosphorylate synthetic polymeric substrates which are coated on an ELISA microtiter plate. The phosphotyrosine produced by src-induced phosphorylation of the polymeric substrate is then quantitated by addition of an anti-phosphotyrosine antibody, the presence of which is detected using a second rabbit anti-mouse antibody which is linked to a reporter enzyme, horseradish peroxidase (HRPO). A similar ELISA assay has been developed by Lazaro et al., which is used for detection of a protein tyrosine kinase. See Lazaro et al., Analytical Biochemistry 192:257-261 (1991). Like the assay of Cleaveland et al., this assay also measures the ability of a protein tyrosine kinase to phosphorylate a synthetic substrate which is bound to microELISA wells.

A direct way to assess specific activation of rPTKs is by analysis of receptor autophosphorylation. See, e.g., Hunter and Cooper Ann Rev Biochem 54:897-930 (1985) and Ullrich and Schlessinger, Cell 61:203-212 (1990). Using this direct approach, Knutson and Buck disclose assays for measuring autophosphorylation of the insulin receptor under *in situ* or *in vitro* conditions (Archives of Biochemistry and Biophysics 285(2):197-204 [1991]). In the *in situ* assay, monolayer cultures of embryonic mouse 3T3-C2 fibroblasts (having the endogenous insulin receptor) are incubated with insulin in large cell culture dishes. Following incubation, the insulin receptor is extracted from the membranes. To achieve extraction of the insulin receptor, the cell monolayers are scraped into a buffer containing protease inhibitors and the cells are then disrupted in a homogenizer. The cellular homogenate is subsequently subjected to centrifugation for 60 min., and the pellet which forms is extracted into buffer containing detergent. Following a further centrifugation step, the supernatant (containing the insulin receptor) is incubated with an anti-insulin receptor antibody. Then, the receptor-antibody complex is incubated with protein A-agarose and unoccupied protein A sites are blocked with normal rabbit IgG. The agarose beads are then centrifuged, the supernatants aspirated and the pellets are re-suspended in buffer containing the radiolabelled anti-phosphotyrosine antibody. The amount of bound iodinated anti-phosphotyrosine antibody is consequently measured.

Klein and his colleagues discuss an assay for measuring insulin activation of the insulin receptor (Klein et al., Diabetes 42:883-890 [1993]). In this assay, aliquots of a heterogeneous population of mononuclear blood cells (including T cells, B cells, macrophages etc) having the insulin receptor are exposed to insulin in centrifuge tubes.

The cells are then lysed in detergent using a motordriven homogenizer and the lysates are concentrated two- to fourfold using vacuum centrifugation. Sometimes, the insulin receptor is also partially purified using wheat germ agglutinin agarose. The supernatants which form following centrifugation, are then transferred to anti-insulin receptor-coated microtiter plates. Insulin (8.7nM) as well as kinase and phosphatase inhibitors are present during receptor immobilization in order to optimize the percentage of receptors captured to the microtiter plates. Activation of the insulin receptor is then measured by transphosphorylation of the substrate Poly-Glu,Tyr with ^{32}P labeled ATP. The supernatants are then spotted onto absorbent paper and the paper is washed with cold TCA to remove unbound ^{32}P -ATP. Remaining ^{32}P -labeled Poly-Glu,Tyr on the washed absorbent paper is subsequently counted by scintillation counting.

Hagino et al. were also interested in studying the insulin receptor in patients (Hagino et al., *Diabetes* 43:274-280 [1994]). As a first step in the assay, Hagino et al. stimulate a primary cell suspension, which is not particularly homogeneous with respect to cell type. In particular, heparinized blood (1ml washed twice with medium and resuspended in 1 ml of medium containing bovine serum albumin, BSA) is exposed to varying concentrations of insulin. The autophosphorylation reaction is stopped, the cells centrifuged for 30 min, the supernatant is discarded and the erythrocyte ghosts thus obtained are resuspended in buffer and centrifuged again. The pellet thereby obtained is adjusted to 500 μl and solubilized in detergent. The solubilized materials are then centrifuged and the resulting supernatant is subjected to sandwich ELISA (using anti-insulin receptor antibodies to capture the insulin receptor) to determine the extent of insulin receptor autophosphorylation.

King et al. in *Life Sciences* 53: 1465-1472 (1993) describe a colorimetric assay for examining inhibitors of the epidermal growth factor (EGF) receptor-associated tyrosine kinase in human intact epidermal A431 cells.

Several others have used an enzyme-conjugated form of the anti-phosphotyrosine antibody in Western blot analyses which measure receptor autophosphorylation. Briefly, Western blotting generally involves electrophoresing activated rPTK on polyacrylamide gel. The rPTK is then transferred to nitrocellulose and immunoblotted with the anti-phosphotyrosine antibody which is labelled to enable detection. See, for example, Wang, *Molecular and Cellular Biology* 5(12):3640-3643 (1985);

Glenney et al., Journal of Immunological Methods 109:277-285 (1988); Kamps, Methods in Enzymology 201:101-110 (1991); Kozma et al., Methods in Enzymology 201:28-43 (1991); Holmes et al., Science 256:1205-10 (1992); and Corfas et al., PNAS, USA 90:1624-1628 (1993). However, with Western blot analysis, accurate quantitation can be very tedious. Furthermore, this technique tends to be time-consuming and generally does not allow high sample throughput.

It is an object of the instant invention to provide a sensitive, reliable assay that measures receptor protein tyrosine kinase (rPTK) autophosphorylation. The assay is desirably useful for qualitatively and quantitatively measuring kinase activation as well as facilitating identification and characterization of potential agonists and antagonists for a selected rPTK. It is a further object of the invention to provide an assay which enables ligand-receptor interactions to be studied for any selected rPTK.

This assay must have a capacity for high throughput, that is, the ability to reliably evaluate large numbers of samples in a relatively short period of time (e.g., in one day). The assay ideally does not use radioactive materials and is also amenable to automation.

It is a further object, in at least one embodiment of the invention, to provide a generic assay which enables a rPTK of interest to be studied, regardless of whether or not a receptor-specific capture agent having the desired characteristics is available. Furthermore, it is an object of the invention to provide an assay which substantially represents the activity of the tyrosine kinase receptor *in situ*. This is desirable insofar as it reduces the possibility that altered interactions between the receptor and the ligand may occur as a consequence of the receptor not being membrane-bound. Furthermore, if the receptor is a multimeric complex, this assay enables the correctly assembled receptor to be studied. It is an additional object to provide a method for measuring serine-threonine kinase phosphorylation, phosphorylation of intracellular kinases and phosphatase activity.

These and other objects will be apparent to the ordinary artisan upon consideration of the specification as a whole.

SUMMARY OF THE INVENTION

Accordingly, the invention provides an assay for measuring activation (i.e., autophosphorylation) of a tyrosine kinase receptor of interest.

The assay can be divided into two major stages, each of which is generally performed in separate assay plates. The first stage of the assay involves activating the receptor and is termed the kinase receptor activation (KIRA) stage of the assay. The second stage of the assay
5 involves measuring receptor activation. Conveniently, this is achieved using an enzyme-linked immunosorbent assay (ELISA) to measure receptor activation.

The KIRA stage of the assay involves activating a tyrosine kinase receptor which is located in the cell membrane of an eukaryotic cell such
10 that the extracellular domain of the receptor faces the external milieu of the cell, the transmembrane domain is located in the cell membrane and the kinase domain is located intracellularly. This stage of the overall assay involves steps (a) to (c) below:

(a) The first solid phase (e.g., a well of a first assay plate) is
15 coated with a substantially homogeneous population of cells (usually a mammalian cell line) so that the cells adhere to the solid phase. Often, the cells are adherent and thereby adhere naturally to the first solid phase. In one embodiment of the invention, the cells have an endogenous tyrosine kinase receptor presented in the cell membrane as discussed above.
20 In an alternative embodiment, the cells have been transformed with DNA encoding a tyrosine kinase receptor or a "receptor construct" defined further below, which DNA is expressed by the cells such that the receptor or receptor construct is suitably positioned in the cell membranes thereof.

The receptor construct comprises a fusion of a kinase receptor and
25 a flag polypeptide. The flag polypeptide is recognized by the capture agent, often a capture antibody, in the ELISA part of the assay. Use of a receptor construct as disclosed herein is particularly advantageous since it provides a "generic" assay wherein autophosphorylation of any tyrosine kinase receptor can be measured, regardless of whether or not a receptor-specific capture agent having the required characteristics is available.
30 Often, the tyrosine kinase receptor is a fusion protein comprising the ECD of a selected tyrosine kinase and the catalytic ICD (and possibly the transmembrane domain) of another well characterized tyrosine kinase (e.g., the Rse receptor).

35 (b) An analyte is then added to the wells having the adhering cells, such that the tyrosine kinase receptor is exposed to (or contacted with) the analyte. This assay enables identification of agonist and antagonist ligands for the tyrosine kinase receptor of interest. In order to detect

the presence of an antagonist ligand which blocks binding and/or activation of the receptor by an agonist ligand, the adhering cells are exposed to the suspected antagonist ligand first and then to the agonist ligand (or to a mixture of the agonist and antagonist) so that competitive inhibition of
5 receptor binding and activation can be measured. Also, the assay can identify an antagonist which binds to the agonist ligand and thereby reduces or eliminates its ability to bind to, and activate, the rPTK. To detect such an antagonist, the suspected antagonist and the agonist for the rPTK are incubated together and the adhering cells are then exposed to this
10 mixture of ligands.

(c) Following exposure to the analyte, the adhering cells are solubilized using a lysis buffer (which has a solubilizing detergent therein) and gentle agitation, thereby releasing cell lysate which can be subjected to the ELISA part of the assay directly, without the need for
15 concentration or clarification of the cell lysate. Thus, this assay provides a significant improvement over assays described by Knutson and Buck, *supra*, Klein et al., *supra*, and Hagino et al., *supra*, insofar as it is surprisingly unnecessary to concentrate the cell lysate prior to the ELISA. Furthermore, unlike the other assays, in the instant assay the cells
20 can be lysed in lysis buffer using gentle agitation without the need for homogenizing, centrifuging or clarifying the cells. The cell lysate thus prepared is then ready to be subjected to the ELISA stage of the assay. It has been discovered that, surprisingly, the first assay plate can be stored at freezing temperatures (i.e., at about -20° to -70°C) for significant
25 periods of time (at least 6 months) before the ELISA stage of the assay. This is a significant finding insofar as the KIRA and ELISA stages of the assay can be performed on separate days.

The ELISA component of the assay comprises steps (d) to (h), described below.

30 (d) As a first step, the second solid phase (usually a well of an ELISA microtiter plate) is coated with a capture agent (often a capture antibody) which binds specifically to the tyrosine kinase receptor, or, in the case of a receptor construct, to the flag polypeptide. Coating of the second solid phase is carried out so that the capture agent adheres to the
35 second solid phase. The capture agent is generally a monoclonal antibody, but, as is described in the examples herein, polyclonal antibodies may also be used.

(e) The cell lysate obtained in step (c) of the above-mentioned KIRA stage of the assay is exposed to, or contacted with, the adhering capture agent so that the receptor or receptor construct adheres to (or is captured in) the second solid phase. Unlike the assay of Klein et al., the instant
5 assay does not require the ligand for the receptor as well as kinase inhibitors to be present to achieve suitable immobilization of the receptor or receptor construct to the second solid phase.

(f) A washing step is then carried out, so as to remove unbound cell lysate, leaving the captured receptor or receptor construct.

10 (g) The adhering or captured receptor or receptor construct is then exposed to, or contacted with, an anti-phosphotyrosine antibody which identifies phosphorylated tyrosine residues in the tyrosine kinase receptor. In the preferred embodiment, the anti-phosphotyrosine antibody is conjugated (directly or indirectly) to an enzyme which catalyses a color
15 change of a non-radioactive color reagent. Accordingly, phosphorylation of the receptor can be measured by a subsequent color change of the reagent. The enzyme can be bound to the anti-phosphotyrosine antibody directly, or a conjugating molecule (e.g., biotin) can be conjugated to the anti-phosphotyrosine antibody and the enzyme can be subsequently bound to the
20 anti-phosphotyrosine antibody via the conjugating molecule.

(h) Finally, binding of the anti-phosphotyrosine antibody to the captured receptor or receptor construct is measured, e.g., by a color change in the color reagent.

The invention also pertains to a Rse.flag reagent which is
25 particularly useful for use in the KIRA ELISA assay. The Rse.flag reagent is a polypeptide comprising a fusion of a flag polypeptide (usually the gD flag described herein) to the carboxyl terminus of the intracellular domain of the Rse rPTK. Generally, the transmembrane domain of Rse and the extracellular domain of another rPTK of interest are also present in the
30 fusion polypeptide reagent. The nucleic acid encoding this reagent and a cell transformed therewith are also claimed.

In yet a further aspect, the invention relates to a kit which can be used in the KIRA ELISA disclosed above which comprises an anti-flag polypeptide capture agent (e.g. a capture antibody) which is usually bound
35 to the second solid phase as described herein. Thus, the kit generally provides an ELISA microtiter plate having an anti-flag polypeptide capture antibody adhering to a well thereof. Optionally, the kit also provides an anti-phosphotyrosine antibody which is often labelled, or reagents for

labelling the anti-phosphotyrosine antibody are supplied with the kit. Sometimes, a homogeneous population of cells which have been transformed with a receptor construct as described herein are also provided with the kit. The kit also suitably includes instructions for carrying out the KIRA
5 ELISA.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1C are diagrammatic representations of Rse.gD (Figure 1A), Receptor ECD/Rse.gD chimera (Figure 1B) and a CHO cell transformed with the
10 Receptor ECD/Rse.gD chimera (Figure 1C).

Figures 2A and 2B depict an alignment of the amino acid sequence (SEQ ID NO: 1) and nucleotide sequence (SEQ ID NO: 2) of Rse.gD. The residues of the signal sequence are indicated with an (*), the transmembrane domain of Rse is boxed and the ECD and ICD of Rse are also delineated. The
15 residues of the gD flag sequence are underlined.

Figure 3 is a flow diagram of an exemplary strategy for selecting a suitable capture agent for use in the assay.

Figure 4 is a flow diagram of an exemplary strategy for selecting a transformed cell suitable for use in the assay, where the cell has a
20 receptor construct with an amino-terminal flag polypeptide located in the cell membrane thereof.

Figure 5 is a flow diagram of an exemplary strategy for selecting a transformed cell suitable for use in the assay, where the cell has a receptor construct with a carboxyl-terminal flag polypeptide located in the
25 cell membrane thereof.

Figure 6 is a flow chart and cartoon illustrating the KIRA ELISA assay for the HER2 receptor described in Example 1.

Figure 7 depicts a p185^{HER2}/HRGB1₁₇₇₋₂₄₄ KIRA ELISA standard curve obtained using the assay described in Example 1. To obtain the standard
30 curve, MCF-7 cells (2×10^5) were stimulated with 3000, 1000, 333, 111, 37, 12, 4, or 0 pM HRGB1₁₇₇₋₂₄₄, as determined by quantitative amino acid analysis (q.a.a.a.). Each calibrator concentration was run in triplicate. The values derived from 10 such standard curves were averaged (total n = 30) and are presented as mean $ABS_{450/650} \pm sd$ vs. HRGB1₁₇₇₋₂₄₄ concentration.

35 Figure 8 depicts heregulin specificity of p185^{HER2}/HRG KIRA ELISA of Example 1. In the assay, MCF-7 cells (2×10^5) were stimulated with either HRGB1₁₇₇₋₂₄₄ (■) at 3000, 1000, 333, 111, 37, 12, 4 or 0 pM or IGF-1 (▲), EGF (□), VEGF (●) or insulin (◆) at 30000, 10000, 3333, 1111, 370, 120, 40 or

0 pM. For all concentrations of ligands, $n = 3$ and data are presented as average $ABS_{450/650} \pm sd$ vs. ligand concentration.

Figure 9 is a flow chart and cartoon illustrating the KIRA ELISA assay for the Rse receptor described in Example 2.

5 Figure 10 depicts a Rse KIRA ELISA standard curve obtained using the assay described in Example 2. To obtain the standard curve, CHO cells transformed with the Rse.gD construct were stimulated with 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200 or 0 diluted, anti-Rse agonist antibody. Each calibrator concentration was run in triplicate. The values are presented
10 as mean $ABS_{450/650} \pm sd$ vs. 1/dilution agonist antibody.

Figure 11 is a flow chart and cartoon illustrating the KIRA ELISA assay for the trk receptors (i.e., trk A, trk B, and trk C) described in Example 3.

Figures 12A-12D depict an alignment of the amino acid sequence
15 (SEQ ID NO: 3) and nucleotide sequence (SEQ ID NO: 4) of gD.trk A used in the assay described in Example 3. The residues of the signal sequence are indicated with an (*), the residues of the gD flag sequence are underlined, the residues of the transmembrane domain of trk A are in bold and the ECD and ICD thereof are also delineated.

20 Figures 13A-13D depict an alignment of the amino acid sequence (SEQ ID NO: 5) and nucleotide sequence (SEQ ID NO: 6) of gD.trk B used in the assay described in Example 3. The residues of the signal sequence are indicated with an (*), the residues of the gD flag sequence are underlined, the residues of the transmembrane domain of trk B are in bold and the ECD
25 and ICD thereof are also delineated.

Figures 14A-14D depict an alignment of the amino acid sequence (SEQ ID NO: 7) and nucleotide sequence (SEQ ID NO: 8) of gD.trk C used in the assay described in Example 3. The residues of the signal sequence are indicated with an (*), the residues of the gD flag sequence are underlined,
30 the residues of the transmembrane domain of trk C are in bold and the ECD and ICD thereof are also delineated.

Figures 15A-15C depict standard curves for trk A, B and C, respectively, which were obtained using the assay described in Example 3. To obtain the standard curves, CHO cells transformed with the gD.trk
35 constructs were stimulated with 3000, 1000, 333, 111, 37, 12, 4 or 0 pM of ligand, i.e. nerve growth factor (NGF, ■), neurotrophin 3 (NT3, ●) or neurotrophin 5 (NT5, ▲). The values are presented as mean $ABS_{450/650} \pm sd$ vs. ligand concentration.

Figures 16A-16L depict the nucleotide sequence (SEQ ID NO: 9) of the pSV117.ID.LL expression vector used for expression of Rse.gD in Example 2.

Figure 17 is a diagrammatic representation of the MPL/Rse.gD chimeric receptor described in Example 4.

5 Figure 18 is a flow chart and cartoon illustrating the KIRA ELISA for the MPL/Rse.gD chimeric receptor described in Example 4.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Abbreviations and Definitions

"rPTK" means a receptor protein tyrosine kinase.

10 "ECD", "TM domain" and "ICD" refer to the extracellular domain, transmembrane domain and intracellular domain of a rPTK, respectively.

"Kinase Receptor Activation" or "KIRA" when used throughout this application refers to the first stage of the instantly claimed assay wherein a cell-bound rPTK is exposed to a potential agonist/antagonist
15 ligand which may (or may not) induce phosphorylation of tyrosine residues in the intracellular domain of the rPTK. The KIRA is generally carried out in the "first assay plate" as defined herein.

"Enzyme-Linked Immunosorbent Assay" or "ELISA" refers to the second stage of the instantly claimed assay and involves measuring tyrosine
20 phosphorylation of the rPTK. The ELISA is normally carried out in the "second assay plate" as disclosed in this application. The ELISA is a "sandwich ELISA" insofar as it involves capturing the rPTK or receptor construct to the second solid phase (usually the well of an ELISA microtiter plate). ELISA assays generally involve the preparation of
25 enzyme-antibody conjugates. The conjugated enzyme cleaves a substrate to generate a colored reaction product that can be detected spectrophotometrically. In this assay, the absorbance of the colored solution in individual microtiter wells is proportional to the amount of phosphotyrosines. A review of ELISA is found in Current Protocols in
30 Molecular Biology, Vol. 2, chapter 11 (1991). While the term "ELISA" is used to describe the second stage of the instant assay, it is only a preferred embodiment of the invention, since, as disclosed herein, techniques other than enzymatic detection are available for measuring binding of the anti-phosphotyrosine antibody to the activated receptor.

35 The terms "receptor", "kinase receptor", "tyrosine kinase", "tyrosine kinase receptor", "receptor protein tyrosine kinase" and "rPTK" are used

interchangeably herein and refer to a protein having at least one phosphate accepting phenolic group. The protein is usually a receptor insofar as it has a ligand-binding ECD, TM domain and ICD. The ICD usually comprises a catalytic kinase domain and has one or more phosphate accepting tyrosine residues. See Figures 1A and 1B, for example. Examples of tyrosine kinase receptors include the insulin receptor, insulin related receptor, epidermal growth factor receptor (EGF-R), platelet-derived growth factor receptors A and B (PDGF-R-A and PDGF-R-B), insulin-like growth factor 1 receptor (IGF-1-R), macrophage colony-stimulating factor receptor (M-CSF-R), HER2/neu/c-erbB-2 receptor, HER3/c-erbB-3 receptor, Xmrk receptor, IRR receptor, fibroblast growth factor (FGF) receptors *bek* and *flg*, c-kit receptor, Flk/kDR receptor, Rse receptor, the *Eph*, *Elk*, *Eck*, *Eek*, *Erk*, *Cek4/Mek4/HEK* and *Cek5* receptors, Axl receptor, hepatocyte growth factor receptor (HGF-R), Flt1 VEGF receptor, SAL-S1 receptor, HpTK 5 receptor, trkA receptor, trkB receptor, and trkC receptor. See, for example, Ullrich and Schlessinger Cell 81:203-212 (1990); Fantl et al., Annu. Rev. Biochem. 62:453-481 (1993); Mark et al., Journal of Biological Chemistry 269(14):10720-10728 (1994); and WO 93/15201.

The terms mentioned above encompass chimeric "receptor" molecules which comprise at least the extracellular domain of a selected tyrosine kinase and the intracellular domain, and optionally, the transmembrane domain of another tyrosine kinase. Of course, the tyrosine kinase of interest can provide the transmembrane domain and/or intracellular domain. The terms also encompass amino acid sequence variants and covalent derivatives of the various rPTKs provided they still display tyrosine kinase phosphorylation activity in the KIRA ELISA. Therefore, the variants will generally have conservative amino acid alterations. The individual domains of the tyrosine kinase can be delineated based on sequence homology to known tyrosine kinases and hydrophobicity plots. For example, the hydrophobic transmembrane domain can be readily determined and the ECD and ICD are usually amino-terminal and carboxyl terminal to the transmembrane domain, respectively. Conveniently, the transmembrane domain and ICD of the Rse receptor can be fused to the ECD of a tyrosine kinase of interest, thereby forming a chimeric receptor which is encompassed by the terms denoting a receptor as mentioned above.

In the preferred embodiment, the rPTK is selected from the group consisting of HER2 receptor (Ullrich and Schlessinger, *supra*), Rse receptor

(Mark et al., supra and SEQ ID NO: 1), trk A receptor (SEQ ID NO: 3), trk B receptor (SEQ ID NO: 5) and trk C receptor (SEQ ID NO: 7).

By "autophosphorylation" is meant activation of the catalytic kinase domain of the rPTK, whereby at least one intrinsic tyrosine residue is phosphorylated. Generally, autophosphorylation will result when an agonist molecule binds to the extracellular domain of the kinase receptor. Without being limited to any particular mechanism of action, it is thought that binding of the agonist molecule may result in oligomerization of the kinase receptor which causes activation of the catalytic kinase domain.

By "solid phase" is meant a non-aqueous matrix to which the cells (in the KIRA stage of the assay) or the capture agent (in the ELISA stage of the assay) can adhere. Usually, the solid phase comprises the well of an assay plate but the invention is by no means limited to this embodiment. For example, the solid phase can comprise a discontinuous solid phase of discrete particles. The particles can be porous and formed from a number of different materials, e.g., polysaccharides (e.g. agarose), polyacrylamides, polystyrene, polyvinyl alcohol, silicones and glasses. For examples of suitable particulate solid phases, see U.S. Patent No. 4,275,149.

By "well" is meant a recess or holding space in which an aqueous sample can be placed. The well is provided in an "assay plate". The invention usually employs a "first assay plate" which is formed from a material (e.g. polystyrene) which optimizes adherence of cells (having the receptor or receptor construct) thereto. Generally, the individual wells of the first assay plate will have a high surface area to volume ratio and therefore a suitable shape is a flat bottom well (where the cells are adherent). The "second assay plate" is generally formed from a material (e.g. polystyrene) which optimizes adherence of the capture agent thereto. The second assay plate may have the same general construction and/or characteristics as the first assay plate. However, separate plates are used for the KIRA stage of the assay and the ELISA stage of the assay.

In the preferred embodiment of the invention, both the first assay plate and the second assay plate are "microtiter" plates. The term "microtiter" plate when used herein refers to an assay plate having between about 30 to 200 individual wells, usually 96 wells. Often, the individual wells of the microtiter plate will hold a maximum volume of about 250 μ l. Conveniently, the first assay plate is a 96 well polystyrene or plastic, cell culture microtiter plate (such as that

sold by Becton Dickinson Labware, Lincoln Park, NJ), which allows for automation. Often, about 50 μ l to 300 μ l, more preferably 100 μ l to 200 μ l, of an aqueous sample comprising cell culture media with the cells suspended therein will be added to each well of the first assay plate in the KIRA
5 stage of the assay. It is desirable to seed between about 1×10^4 to 3×10^5 cells per well. More preferably, 5×10^4 to 1×10^5 cells per well are seeded. Usually, the second assay plate will comprise a polystyrene microtiter ELISA plate such as that sold by Nunc Maxisorp, Inter Med, Denmark.

10 The term "homogeneous population of cells" refers to a substantially homogeneous population of cells wherein at least about 80%, and preferably about 90%, of the cells in the population are of the same cell type. Therefore, it is convenient to use a cell line. The cell line is a eukaryotic cell line, normally an animal cell line and desirably a
15 mammalian cell line.

The cells have, or are transformed to produce, the selected receptor or a receptor construct. For example, where the kinase receptor is known to be present in a certain cell line (e.g., the HER2 receptor in the MCF-7 cell line) no transformation step is required. Conversely, it may be
20 necessary to transform a cell with a nucleic acid encoding the receptor, where the cell does not make the receptor, or does not have suitable numbers of the receptor in the cell membrane thereof. Accordingly, the cell is transformed with a nucleic acid encoding the receptor (or receptor construct) and the nucleic acid is expressed so that the ECD of the
25 receptor faces the external milieu of the cell, the transmembrane domain is located in the cell membrane and the kinase domain is located intracellularly.

Where the assay relies on activating the endogenous rPTK, a cell line is selected which is known to produce the rPTK of interest, provided
30 sufficient levels of the rPTK are present in the cell membrane thereof to enable detection. As a general proposition, a minimum number of about 1×10^4 receptors/cell is required. For example, the MCF-7 cell line (ATCC-HTB 22) which produces the HER2 receptor was shown to be useful in the assay. There are 5×10^4 HER2 receptors/MCF-7 cell. Examples of other cell lines
35 and their respective rPTKs include, embryonic mouse 3T3-C2 fibroblast cell line and the insulin receptor, and Hep 3B (ATCC # HB 8064) cell line and the Rse receptor. However, the degree of expression of the rPTK nucleic acid in the cell line is not so high that it results in constitutive

phosphorylation of the rPTK. For example, the SK-BR-3 cell line (ATCC HTB30), which has 3×10^6 HER2 receptors/cell, was found to be unsuitable for use in the assay disclosed herein. Therefore, it may be useful to use a cell line which has less than about 3×10^6 receptors/cell, depending on the type of receptor. The number of receptors/cell can be measured using
5 Scatchard analysis, for example (Scatchard, Ann. NY Acad. Sci. 51:660-672 [1949]; and Goodwin et al., Cell 73:447-456 [1993]). However, selection of a cell line having a suitable number of receptors/cell is possible using the techniques described herein.

10 The term "adherent" when used herein to describe the cell, refers to a cell which naturally adheres to the first solid phase (often the well of the first assay plate), thereby forming a fairly uniform coating of the cells on the inside surface of the well. The uniform coating of cells generally forms following incubation of the cells in the wells of the first
15 assay plate for about 8-16 hours. After incubation, non-adhering cells and cell culture medium are decanted off the first assay plate. Incubation is usually carried out at a temperature which is optimal for cell growth, i.e., about 37°C. Examples of adherent cell lines include CHO cells (Urlaub and Chasin, Proc. Natl. Acad. Sci. USA 77:4216 [1980]), MCF-7 cells (ATCC HB
20 22), 293 cells (Graham et al., J. Gen. Virol. 36:59 [1977]), Swiss albino 3T3 fibroblast cell line (ATCC No. CCL 92) and U937 macrophage cell line (ATCC No. CRL 1593).

A "flag polypeptide" comprises a short polypeptide which has enough residues to provide an epitope (preferably a linear epitope) against which
25 a "capture agent" thereagainst can be made, yet is short enough such that it does not interfere with activity of the rPTK. The flag polypeptide is also sufficiently unique so that the capture agent thereagainst does not bind to other reagents in the assay. Selection of a "unique" flag polypeptide sequence can be accomplished by comparing the sequence of a
30 proposed flag polypeptide against other known sequences in Genbank or EMBL, for example. Suitable flag polypeptides generally have at least 6 amino acid residues and usually between about 8-80 amino acid residues (preferably between about 9-30 amino acid residues).

By "receptor construct" is meant a polypeptide which comprises a
35 fusion of a kinase receptor and a flag polypeptide as defined above. The flag polypeptide is provided at a location in the receptor construct such that: a) the flag polypeptide does not interfere with ligand binding to the receptor; b) the flag polypeptide does not interfere with

autophosphorylation of the receptor and c) the flag polypeptide is presented in a suitable configuration so that it can bind to the capture agent in the ELISA stage of the assay. Often, the polypeptide flag will be present at the N-terminus of the receptor construct. See, for example, Example 3 which refers to the gD.trk constructs. Alternatively, the flag polypeptide may be present at the C-terminus of the receptor construct. See, for example, Example 2 which refers to the Rse.gD construct. See also Figures 1A-1C. The Rse construct disclosed herein is particularly useful, since the ICD (and optionally the transmembrane domain) thereof can be fused to the ECD of a kinase receptor of interest, thereby obviating the need to establish where the flag polypeptide should be located with respect to the kinase receptor of interest.

"Rse.gD" refers to a receptor construct which is the Rse receptor protein tyrosine kinase with the Herpes Simplex virus glycoprotein D (gD) flag polypeptide fused to the COOH-terminus thereof.

"Rse.flag reagent" refers to a polypeptide which comprises the ICD of the Rse receptor fused at its COOH-terminus to a flag polypeptide (normally the gD flag polypeptide). Sometimes, the TM domain of Rse and the ECD of a rPTK of interest will also be present in the Rse.gD. reagent. "Receptor ECD/Rse.gD Chimera" refers to a fusion of the ECD of a rPTK of interest to the TM and ICD domains of Rse which are fused COOH-terminally to the gD flag polypeptide.

"gD.trkA", "gD.trkB" and "gD.trkC" refer to each of the trk receptors (A-C) having the gD flag polypeptide fused to the amino-termini thereof. By "capture agent" is meant a compound or agent which is able to adhere to the second solid phase, as herein defined, and which is selective for a rPTK or receptor construct. Thus, the capture agent captures the receptor or receptor construct to the wells of the second assay plate. Usually, the capture agent binds selectively to the flag polypeptide which has been fused to the receptor of interest. Binding of the capture agent is not affected by the presence or absence of ligand bound to the receptor and does not induce receptor activation upon capture. Furthermore, the capture agent does not sterically block access to the phosphorylated tyrosine(s) by the anti-phosphotyrosine antibody. Means for selecting suitable capture agents are described herein. Generally, the capture agent will comprise an antibody (e.g., an affinity purified polyclonal antibody or a monoclonal antibody), but other selective agents, such as streptavidin which binds selectively to the "strep-tag" polypeptide can also be used

(see Schmidt et al., Protein Engineering 6(1):109-122 [1993]). Streptavidin can be purchased commercially from Zymed Laboratories, S. San Francisco, CA, for example. Alternatively, the capture agent can comprise protein A (which binds specifically to immunoglobulins). In this
5 embodiment of the invention, the activated receptor or receptor-construct present in the cell lysate is incubated with an antibody which binds specifically thereto, thereby forming a receptor-antibody complex. This complex can be captured by protein A by virtue of its specific binding to the antibody present in the complex. Protein A can be purchased
10 commercially from Pharmacia Biotech, Inc., Piscataway, New Jersey, for example. A strategy for selecting a suitable capture agent is depicted in Figure 3 and will be described in more detail later herein.

In the most preferred embodiment, the capture agent is a monoclonal antibody which binds specifically to a flag polypeptide (which is present
15 in the receptor construct). Examples of suitable flag polypeptides and their respective capture antibodies include the flu HA flag and its antibody 12CA5, (Field et al., Mol. Cell. Biol. 8:2159-2165 [1988]); the c-myc flag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan et al., Molecular and Cellular Biology 5(12):3610-3616 [1985]); as well as
20 the Herpes Simplex virus glycoprotein D (gD) flag and the 5B6 antibody thereto (Paborsky et al., Protein Engineering 3(6):547-553 [1990] and Mark et al., Journal of Biological Chemistry 269(14):10720-10728 [1994]). Other flag polypeptides have been disclosed. Examples include the Flag-peptide (Hopp et al., BioTechnology 6:1204-1210 [1988]); the KT3 epitope peptide
25 (Martin et al., Science 255:192-194 [1992]); an α -tubulin epitope peptide (Skinner et al., J. Biol. Chem 266:15163-15166 [1991]); and the T7 gene 10 protein peptide tag (Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA 87:6393-6397 [1990]). Once the flag polypeptide has been selected as discussed above, a capture antibody thereto can be generated using the
30 techniques disclosed herein.

The term "analyte" refers to a compound or composition to be studied, usually to investigate its ability to activate (or prevent activation of) the tyrosine kinase receptor of interest. The analyte can comprise a bodily fluid (such as plasma or amniotic fluid) or a composition known to
35 contain, or suspected of containing, a ligand for the tyrosine kinase receptor. The analyte can also comprise a cell which has a ligand to the rPTK of interest.

"Ligand" when used herein refers to a molecule which is able to bind to the ECD of the tyrosine kinase of interest or to a known agonist for the tyrosine kinase of interest. The ligand will usually be an agonist or antagonist for the tyrosine kinase.

5 By "agonist" is meant a molecule which is able activate the intracellular kinase domain of the tyrosine kinase upon binding to the ECD. Often, the agonist will comprise a growth factor (i.e., a polypeptide that is able to stimulate cell division). Exemplary growth factors include heregulin (HRG), insulin, insulin-like growth factors I and II (IGF-I and
10 IGF-II), epidermal growth factor (EGF), interleukins (e.g., IL-8), macrophage colony-stimulating factor (M-CSF), erythropoietin (EPO), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factors alpha and beta (TGF- α and TGF- β), hepatocyte growth factor (HGF), and nerve growth factor (NGF). Alternatively, the
15 agonist can be an antibody against the rPTK (see, e.g., Yarden, Proc. Natl. Acad. Sci. USA 87:2569-2573 [1990]). However, other non-protein agonists such as small organic molecules are also encompassed by the invention.

By "antagonist" is meant a molecule which blocks agonist action. Usually, the antagonist will either: (a) bind to the rPTK and thereby
20 block binding and/or activation of the rPTK by an agonist thereto (the antagonist may bind to the ECD of the rPTK, but this is not necessarily the case) or (b) bind to the agonist and thus prevent activation of the rPTK by the agonist. This assay facilitates the detection of both types of antagonist. The antagonist may, for example, comprise a peptide fragment
25 comprising the receptor binding domain of the endogenous agonist ligand for the receptor. The antagonist may also be an antibody which is directed against the ECD of the rPTK, or against a known agonist for the rPTK. However, other non-protein molecules are also encompassed by this term.

The term "antibody" is used in the broadest sense and specifically
30 covers monoclonal antibodies and antibody compositions with polyeptitopic specificity (i.e. polyclonal antibodies). The polyclonal antibodies are preferably "affinity purified" antibodies. The term "affinity purified" means that the antibodies have been purified using the antigen (e.g. the rPTK or fragment thereof or the flag polypeptide) to selectively purify the
35 polyclonal antibodies. Affinity purification can be achieved by immobilizing the antigen on an affinity column (e.g. an agarose column) and passing the polyclonal antibodies through the column. The affinity purified antibodies can be subsequently eluted from the column by changing

the elution conditions or by adding a chaotropic agent, for example. For a review of affinity purification techniques with respect to antibodies, see Current Protocols in Immunology, Ed. Coligen et al., Wiley publishers, Vols. 1 and 2, for example.

5 The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against
10 a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

15 The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of a selected antibody with a constant domain (e.g. "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous
20 proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, F(ab')₂, and Fv), so long as they exhibit the desired biological activity. [See, e.g. U.S. Patent No. 4,816,567 and Mage & Lamoyi, in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc., New
25 York (1987))].

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies
30 to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler & Milstein, Nature, 256: 495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al.,
35 Nature, 352: 624-628 (1991) and Marks et al., J. Mol. Biol., 222: 581-597 (1991), for example.

The term "anti-phosphotyrosine antibody" refers to a molecule, usually an antibody, which binds selectively to phosphorylated tyrosine

residues in the kinase domain of a rPTK. The antibody can be polyclonal, but is desirably a monoclonal antibody. Anti-phosphotyrosine polyclonal antibodies can be made using the techniques disclosed in White and Backer, Methods in Enzymology 201:65-67 [1991] and monoclonal anti-phosphotyrosine
5 antibodies can be obtained commercially from Upstate Biologicals, Inc. (UBI, Lake Placid, NY), for example.

The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly with a molecule (such as the anti-phosphotyrosine antibody). The label may be detectable
10 by itself (e.g. radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze a chemical alteration of a substrate compound or composition which is detectable. The preferred label is an enzymatic one which catalyzes a color change of a non-radioactive color reagent.

15 By "washing" is meant exposing the solid phase to an aqueous solution (usually a buffer or cell culture media) in such a way that unbound material (e.g., non-adhering cells, non-adhering capture agent, unbound ligand, receptor, receptor construct, cell lysate, or anti-phosphotyrosine antibody) is removed therefrom. To reduce background noise, it is
20 convenient to include a detergent (e.g. Triton X) in the washing solution. Usually, the aqueous washing solution is decanted from the wells of the assay plate following washing. Conveniently, washing can be achieved using an automated washing device. Sometimes, several washing steps (e.g., between about 1 to 10 washing steps) may be required.

25 By "block buffer" is meant an aqueous, pH buffered solution containing at least one blocking compound which is able to bind to exposed surfaces of the second solid phase which are not coated with capture agent. The blocking compound is normally a protein such as bovine serum albumin (BSA), gelatin, casein or milk powder and does not cross-react with any of
30 the reagents in the assay (e.g., the anti-phosphotyrosine antibodies and detection reagents). The block buffer is generally provided at a pH between about 7 to 7.5 and suitable buffering agents include phosphate and TRIS.

By "lysis buffer" is meant an aqueous, pH buffered solution
35 comprising a solubilizing detergent, one or more protease inhibitors and at least one phosphatase inhibitor (such as sodium orthovanadate). The term "solubilizing detergent" refers to a water miscible, non-ionic detergent which lyses cell membranes of eukaryotic cells but does not

denature or activate the receptor or receptor construct. Examples of suitable non-ionic detergents include Triton-X 100, Tween 20, CHAPS and Nonidet P-40 (NP40) available from Calbiochem, La Jolla, California, for example. Many other non-ionic detergents are available in the art. Examples
5 of suitable protease inhibitors include phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin, aprotinin, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride-bestatin, chymostatin and benzamidine. Preservatives (e.g., thimerosal) and one or more compounds which maintain the isotonicity of the solution (e.g., sodium chloride [NaCl] or sucrose)
10 and a buffer (e.g., Tris or PBS) are usually also present. Generally, the pH of the lysis buffer is in the range about 7 to 7.5.

Usually, following addition of the lysis buffer to the first assay plate, the first assay plate is "gently agitated" and this expression refers to the act of physically shaking the first assay plate (normally
15 using a circular motion) at a substantially low velocity. Gentle agitation does not involve mechanically disrupting the cells (e.g. by homogenizing or centrifuging the cells). Exemplary shaking velocities are in the order of 200 to 500 rpm, preferably 300 to 400 rpm in a Bellco orbital shaker, for example.

20 II. Modes for Practicing the Invention

1. Kinase Receptor Activation - KIRA

The first stage of the assay involves phosphorylation of the kinase domain of a kinase receptor, wherein the receptor is present in the cell membrane of a eukaryotic cell. The receptor may be an endogenous receptor
25 or nucleic acid encoding the receptor may be transformed into the cell. In one embodiment of the invention, nucleic acid encoding a receptor construct is transformed into the cell. Exemplary techniques for transforming the cell with either the receptor or the receptor construct nucleic acid follow.

30 A. Transformation of the cells

The instant invention provides a substantial improvement over soluble kinase receptor assays insofar as it is considered to more accurately reflect the activity of the receptor *in situ*. It has been discovered that it is possible to transform eukaryotic cells with a receptor construct
35 (comprising the kinase receptor and either an amino- or carboxyl-terminal flag polypeptide) so that the receptor construct assembles itself appropriately in the cell membrane and still retains tyrosine kinase

activity which can be detected in the ELISA stage of the assay. This provides a generic assay for measuring tyrosine kinase activity of any tyrosine kinase of interest.

If a suitable capture agent as described herein is available for a selected rPTK, cells can be transformed with the nucleic acid encoding the receptor alone, without the flag polypeptide. Alternatively, if cells are available which produce the receptor (e.g., MCF-7 cells which produce the HER2 receptor), it is not necessary to transform the cells for use in the assay.

In order to transform the cells with the nucleic acid encoding the rPTK or receptor construct, nucleic acid encoding the rPTK and, optionally, the flag polypeptide, is isolated. This can be achieved by screening a cDNA or genomic library known to contain the DNA encoding the rPTK or flag polypeptide of interest with a selected labelled probe (e.g., an antibody or oligonucleotide- probe) for the rPTK or flag polypeptide, using standard procedures as described in chapters 10-12 of Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989), for example. Alternatively, the nucleic acid encoding the flag polypeptide can be made synthetically using an oligo-synthesizing machine (Applied Biosystems, CA). An alternative means to isolate the nucleic acid encoding the rPTK or flag polypeptide is to use PCR methodology as described in section 14 of Sambrook et al., *supra*. Isolation of only the ECD of the rPTK of interest is required, since this nucleic acid can be fused to the nucleic acid encoding the TM and ICD of the Rse-flag polypeptide construct disclosed herein. See Figures 1A - 1C and SEQ ID NOS: 1 and 2. If necessary however, conventional primer extension procedures as described in section 7.79 of Sambrook et al., *supra*, can be used to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

A preferred method of practicing this invention is to use carefully selected oligonucleotide sequences to screen cDNA libraries from various tissues, preferably mammalian cell lines having the rPTK of interest. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized.

The oligonucleotide must be labeled such that it can be detected upon hybridization to DNA in the library being screened. The preferred method of labeling is to use ³²P- labeled ATP with polynucleotide kinase, as is well known in the art, to radiolabel the oligonucleotide. However, other

methods may be used to label the oligonucleotide, including, but not limited to, biotinylation or enzyme labeling.

In order to provide nucleic acid encoding a receptor construct, nucleic acid encoding the rPTK is fused at its 3' end to nucleic acid
5 encoding the N-terminus of the flag polypeptide. Alternatively, the nucleic acid encoding the rPTK will be fused at its 5' end to nucleic acid encoding the carboxyl terminus of the flag polypeptide. Thus, the flag polypeptide is provided at either the carboxyl- or amino- terminus of the receptor construct. Examples of suitable flag polypeptides are provided above.
10 Selection of other suitable flag polypeptides is possible using the techniques described herein.

In order to generate fusions between the Rse.flag reagent and a rPTK of interest, the nucleic acid encoding the ECD of the rPTK of interest is fused at its 3' end to the nucleic acid encoding the amino terminus of the
15 Rse.flag reagent.

The nucleic acid (e.g., cDNA or genomic DNA) encoding the rPTK or receptor construct is then inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available to the skilled practitioner but must be compatible with the cell
20 which is to be used in the assay. The vector will have vector components the presence of which will depend on various factors. Such components include, for example, a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Selection of these vector components shall be
25 described below.

Incorporation of a signal sequence into the expression vector is required since the rPTK or receptor construct must be transported to the cell membrane where it is positioned such that the ECD faces the external milieu of the cell. Therefore, a signal sequence suitable for positioning
30 the rPTK or receptor construct in such a manner is used. The signal sequence is generally a component of the vector, or it may be a part of the rPTK or receptor construct DNA that is inserted into the vector. If a heterologous signal sequence is used, it is from those that are recognized and processed (i.e., cleaved by a signal peptidase) by the host cell.

35 For yeast secretion the native signal sequence may be substituted by, e.g., the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces* α -factor leaders, the latter described in U.S. Patent No. 5,010,182 issued 23 April 1991), or acid phosphatase

leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cells expression of the DNA encoding the native signal sequence (e.g., the rPTK pre-sequence that normally directs secretion of rPTK from mammalian cells *in vivo*) is satisfactory, although other mammalian signal sequences may be suitable, such as signal sequences from other animal rPTKs, and signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex gD signal.

10 The DNA for such precursor region is ligated in reading frame to DNA encoding the rPTK or receptor construct.

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. The 2 μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e., they are capable of replication in at least one class of organisms but can be transformed into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transformed into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transformation of *Bacillus* with this vector results in homologous recombination with the genome and insertion of rPTK or receptor construct DNA. However, the recovery of genomic DNA encoding the rPTK or receptor construct is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the rPTK or receptor construct DNA.

Expression and cloning vectors usually contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the

survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other
5 toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a
10 heterologous gene express the DNA encoding a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern et al., J. Molec. Appl. Genet. 1:327 [1982]), mycophenolic acid (Mulligan et al., Science 209:1422 [1980]) or hygromycin (Sugden et al., Mol. Cell. Biol. 5:410-413 [1985]).
15 The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the
20 rPTK or receptor construct nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection
25 agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes the rPTK or receptor construct. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of
30 recombinant cells. Increased quantities of rPTK or receptor construct are synthesized from the amplified DNA. Other examples of amplifiable genes include metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, etc.

For example, cells transformed with the DHFR selection gene are first
35 identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and

propagated as described by Urlaub and Chasin, Proc. Natl. Acad. Sci. USA 77:4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding the rPTK or receptor construct. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060).

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding the rPTK or receptor construct, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 (Stinchcomb et al., Nature 282:39 [1979]; Kingsman et al., Gene 7:141 [1979]; or Tschemper et al., Gene 10:157 [1980]). The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, Genetics 85:12 [1977]). The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

In addition, vectors derived from the 1.6 μ m circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts. Bianchi et al., Curr. Genet. 12:185 (1987). More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis*. Van den Berg, Bio/Technology 8:135 (1990). Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed. Fleer et al., Bio/Technology 9:968-975 (1991).

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the rPTK or receptor construct nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally

within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the rPTK nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to rPTK or receptor construct-encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native rPTK promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the rPTK or receptor construct DNA. The promoter will be one which results in the accumulation of suitable numbers of receptor or receptor construct in the cell membrane of the transformed cell (i.e. so that autophosphorylation of the receptor is detectable in the ELISA but constitutive phosphorylation does not occur). Selection of a suitable promoter to achieve this is possible following the guidelines herein for selecting cells for use in the KIRA ELISA.

Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem. 255:2073 [1980]) or other glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg., 7:149 [1968]; and Holland, Biochemistry 17:4900 [1978]), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are

the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and
5 promoters for use in yeast expression are further described in Hitzeman et al., EP 73,657A. Yeast enhancers also are advantageously used with yeast promoters.

rPTK or receptor construct transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the
10 genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin
15 promoter, from heat-shock promoters, and from the promoter normally associated with the rPTK or receptor construct sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral
20 origin of replication. Fiers et al., Nature 273:113 (1978); Mulligan and Berg, Science 209:1422-1427 (1980); Pavlakis et al., Proc. Natl. Acad. Sci. USA 78:7398-7402 (1981). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. Greenaway et al., Gene 18:355-360 (1982). A system for
25 expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. 4,419,446. A modification of this system is described in U.S. 4,601,978. See also Gray et al., Nature 295:503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes et al., Nature 297:598-601 (1982) on expression of human β -interferon cDNA
30 in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA 79:5166-5170 (1982) on expression of the human interferon β 1 gene in cultured mouse and rabbit cells; and Gorman et al., Proc. Natl. Acad. Sci. USA 79:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney
35 cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

Transcription of DNA encoding the rPTK or receptor construct by higher eukaryotes may be increased, if increased numbers of the rPTK or receptor construct per cell are required to facilitate detection in the ELISA stage of the assay. This may be achieved by inserting an enhancer
5 sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' (Laimins et al., Proc. Natl. Acad. Sci. USA 78:993 [1981]) and 3' (Lusky et al., Mol. Cell Bio. 3:1108 [1983]) to
10 the transcription unit, within an intron (Banerji et al., Cell 33:729 [1983]), as well as within the coding sequence itself (Osborne et al., Mol. Cell Bio. 4:1293 [1984]). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus.
15 Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into
20 the vector at a position 5' or 3' to the rPTK or receptor construct-encoding sequence, but is preferably located at a site 5' from the promoter.

Expression vectors used in eukaryotic host cells will also contain sequences necessary for the termination of transcription and for
25 stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the rPTK or receptor construct.

30 Construction of suitable vectors containing one or more of the above listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed,
35 the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced

by the method of Messing et al., Nucleic Acids Res. 9:309 (1981) or by the method of Maxam et al., Methods in Enzymology 65:499 (1980).

Other methods, vectors, and host cells suitable for adaptation to the synthesis of the rPTK or receptor construct in recombinant vertebrate cell culture are described in Gething et al., Nature 293:620-625 (1981); Mantei
5 et al., Nature 281:40-46 (1979); Levinson et al.; EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression of rPTK or receptor construct DNA is pRK5 (EP 307,247) or pSV16B (PCT pub. no. WO 91/08291 published 13 June 1991).

10 Examples of suitable eukaryotic cell lines for transformation include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* (Beach and Nurse, Nature 290:140 [1981]; EP 139,383 published 2 May 1985); *Kluyveromyces* hosts (U.S. 4,943,529; Fleer et al., Bio/Technology 9:968-975 [1991]) and *Aspergillus* hosts such as *A. nidulans* (Ballance et al., Biochem. Biophys.
15 Res. Commun. 112:284-289 [1983]; Tilburn et al., Gene 26:205-221 [1983]; Yelton et al., Proc. Natl. Acad. Sci. USA 81:1470-1474 [1984]) and *A. niger* (Kelly and Hynes, EMBO J. 4:475-479 [1985]), among lower eukaryotic host microorganisms.

Examples of useful animal host cell lines for transformation include
20 monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen. Virol. 36:59 [1977]); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA 77:4216 [1980]); mouse sertoli cells
25 (TM4, Mather, Biol. Reprod. 23:243-251 [1980]); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse
30 mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci. 383:44-68 [1982]); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient
35 media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or as a chromosomal

integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. Successful transformation is generally recognized when any indication of the operation of this vector occurs within the host cell.

5 For mammalian cells, the calcium phosphate precipitation method of Graham and Van der Eb, Virology 52:456-457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. 4,399,216 issued 16 August 1983. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. Bact. 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA) 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, etc., may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology (1989), Keown et al., Methods in Enzymology 185:527-537 (1990), and Mansour et al., Nature 336:348-352 (1988).

The mammalian host cells used to produce the rPTK or receptor construct may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, Meth. Enz. 58:44 (1979), Barnes and Sato, Anal. Biochem. 102:255 (1980), U.S. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Patent Re. 30,985; or U.S. Patent No. 5,122,469, the disclosures of each of which are incorporated herein by reference, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed., IRL Press, 1991.

5 Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA (Thomas, Proc. Natl. Acad. Sci. USA 77:5201-5205 [1980]), dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe, based on the sequences
10 provided herein. Various labels may be employed, most commonly radioisotopes, particularly ^{32}P . However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as
15 radionuclides, fluorophores, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the
20 formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining to quantitate directly the expression of gene product.

25 B. *Selecting cells for use in the assay*

As mentioned above, the cells to be subjected to the assay can be (a) cells having an endogenous receptor, (b) cells which have been transformed with a rPTK, or (c) cells transformed with a receptor construct. The suitability of the cells for use in the assay is investigated.

30 Cells having the endogenous rPTK can be subjected to a test-run KIRA ELISA using a known ligand to the PTK (e.g. an agonist antibody) and a control (e.g. the diluent for the agonist antibody). A range of ligand concentrations such as those used herein (see Examples 1, 2 and 3) will be used to determine whether sufficient numbers of the receptor are present
35 in the cells being tested. In order to discover whether a cell line is unsuitable because the receptor is constitutively phosphorylated, the cell line can be subjected to the KIRA ELISA disclosed herein, wherein it is exposed to both positive and negative controls (e.g. a known agonist ligand

in cell culture media as described herein as a positive control and the cell culture media without the agonist ligand as the negative control). If phosphorylation of the receptor is detected for both positive and negative controls, this may be indicative that constitutive phosphorylation of the receptor is occurring. However, it is possible that a constituent of the serum in the cell culture media is activating the receptor. Thus, the cells can be "starved" in serum-free media for about 2-12 hours (depending on cell survival) and then the assay is repeated using the positive and negative controls. If activation is detected for both controls, the cell line may be considered unsuitable and another cell line can be tested.

If the cell line is transformed with the receptor (without the flag polypeptide) a strategy similar to that depicted in Figure 4 can be used to discover whether or not the cell line is suitable for use in the assay. As a first step, successful transformation and expression of the nucleic acid encoding the rPTK is determined (see Figure 4, step b). In order to identify whether the ECD of the rPTK is present on the surface of the cells, flow cytometric analysis can be performed using an antibody to the ECD of the receptor. The antibody can be made using the techniques for generating antibodies discussed herein. Flow cytometric analysis can be carried out using the techniques described in Current Protocols in Immunology, Ed. Coligen et al., Wiley publishers, Vols. 1 and 2, for example. Briefly, flow cytometric analysis involves incubating intact cells (having the receptor) with antibodies to the ECD thereof, followed by washing. The antibody-bound cells are then incubated with species specific anti-antibody antibodies conjugated to a fluorochrome. Following washing, the labeled cells are analyzed by fluorescence-activated flow cytometry to detect whether the ECD is present on the surface of the cells.

In the following step, i.e. Figure 4, step (c), the ability of the cell-bound receptor to be activated is tested. In order to determine this, the transformed cells are exposed to a known agonist to the receptor (e.g. the endogenous ligand or an agonist antibody for the receptor). Following exposure, the cells are lysed in a suitable buffer (e.g. sodium dodecylbenzenesulfonate in phosphate buffered saline; SDS in PBS) and subjected to Western blotting with anti-phosphotyrosine antibodies as described in Wang, Molecular and Cellular Biology 5(12):3640-3643 (1985); Glenney et al., Journal of Immunological Methods 109:277-285 (1988); Kamps, Methods in Enzymology 201:101-110 (1991); Kozma et al., Methods in

Enzymology 201:28-43 (1991); Holmes et al., Science 256:1205-10 (1992); or Corfas et al., PNAS, USA 90:1624-1628 (1993), for example.

Assuming the Western blotting step indicates that the rPTK can be activated, a KIRA ELISA test run can be performed, see Figure 4 step (d),
5 to further establish whether or not the transformed cell line can be used in the assay.

In the preferred embodiment of the invention, the KIRA ELISA is a "generic" assay insofar as any rPTK of interest can be studied regardless of the availability of receptor-specific reagents (i.e., capture agent).
10 This embodiment employs a receptor construct having a flag polypeptide at either the amino or carboxyl terminus of the receptor.

If the flag polypeptide is provided at the NH₂-terminus (see, e.g., the gD.trk A, B and C receptor constructs disclosed in Example 3), the procedure for selecting a transformed cell line for use in the assay
15 summarized in Figure 4 can be performed. In this embodiment, the cells are transformed with the flag polypeptide-receptor construct as described earlier herein. See step (a). In step (b), successful transformation of the receptor and flag polypeptide (i.e. the receptor construct) is confirmed. In order to study this, two-dimensional flow cytometric analysis can be
20 performed using antibodies to both the flag polypeptide and the ECD of the receptor. Techniques for two-dimensional flow cytometric analysis are disclosed in Current Protocols in Immunology, supra. Assuming successful transformation of the receptor construct is demonstrated, steps (c) and (d) of Figure 4 are then performed. See the discussion above, for an
25 explanation of steps (c) to (d) of Figure 4.

A technique for identification of cells which have been successfully transformed with the receptor construct having a C-terminal flag polypeptide and which cells are also suitable for use in the assay is illustrated in Figure 5. Following cell transformation [step (a)],
30 successful transformation of the receptor is determined by flow cytometric analysis using an antibody directed against the ECD of the receptor of interest, for example. Flow cytometric analysis can be performed substantially as described above. This forms step (b) of the procedure outlined in Figure 5.

35 Following step (b), successful transformation of the entire receptor construct (including the COOH-terminal flag polypeptide) is analyzed in step (c). This can be achieved by lysing the cells (using techniques for lysing cells disclosed herein) and immunoprecipitating the membrane extract

with an antibody against the receptor of interest. This immunoprecipitated membrane extract is then subjected to Western blot analysis with antibodies specific for the flag polypeptide. Alternatively, rPTK-specific ELISA analysis of anti-flag polypeptide captured membrane lysate can be carried
5 out. Briefly, this involves coating ELISA wells with appropriate flag specific capture agent. The wells are blocked, washed, and the lysate is then incubated in the wells. Unbound receptor construct is removed by washing. The wells are then reacted with receptor-specific antibody or antibodies, either directly or indirectly conjugated to HRPO. The wells
10 are washed and the HRPO is then exposed to the chromogenic substrate (e.g., TMB).

Steps (d) and (e), i.e., detecting receptor activation and KIRA ELISA test run, are essentially the same as those steps described above.

Once useful cells are identified, they are subjected to the KIRA
15 stage of the instantly claimed assay.

C. Coating the first solid phase with the cells

The first solid phase (e.g. a well of a first assay plate) is coated with cells having the endogenous receptor or cells which have been transformed pursuant to the preceding sections.

20 Preferably, an adherent cell line is chosen, so that the cells naturally adhere to the first solid phase. However, use of an adherent cell line is not essential. For example, non-adherent cells (e.g. red blood cells) can be added to round bottomed wells of an assay plate such as that sold by Becton Dickinson Labware, Lincoln Park, New Jersey, for example.
25 The assay plate is then placed in a plate carrier and centrifuged so as to create a pellet of cells adhering to the base of the wells. The cell culture supernatants are removed using a pipette. Thus, use of an adherent cell is clearly advantageous over non-adherent cells since it reduces variability in the assay (i.e, the cells in the pellet of the round bottom
30 wells may be taken up with the supernatant when the alternative method is used).

The cells to be added to the wells of the first assay plate may be maintained in tissue culture flasks and utilized when cells densities of about 70-90% confluency are achieved. Then, generally between about 1×10^4 to 3×10^5 (and preferably 5×10^4 to 1×10^5) cells are seeded per
35 flat-bottom well, using a pipette, for example. It has been found that, contrary to expectations, addition of cell concentrations mentioned above is sufficient to enable activation of the rPTK to be measured in the ELISA

stage of the assay, without the need to concentrate or clarify the cells or cell lysate prior thereto. Often, the cells are diluted in culture medium prior to seeding them in the wells of the microtiter plate to achieve the desired cell densities.

- 5 Usually, the cells are cultured in the microtiter plates for a sufficient period of time to optimize adherence to the wells thereof, but not too long such that the cells begin to deteriorate. Thus, incubation for about 8 to 16 hours at a temperature which is the physiological optimum for the cells (usually about 37°C) is preferred. Suitable media for
10 culturing the cells are described in Section 1A above. Culturing in 5% CO₂ is recommended.

- Following incubation overnight, the well supernatants are decanted and excess supernatant may be further removed by lightly tamping the microtiter plates with an absorbent substrate, e.g., a paper towel, but a
15 sponge works equally well. Thus, a substantially homogeneous layer of adhering cells remains on the internal surfaces of the individual wells of the microtiter plate. These adhering cells are then exposed to the analyte.

D. Preparation and addition of the analyte

- 20 As mentioned above, the analyte may comprise an agonist ligand (or suspected agonist) or an antagonist (or suspected antagonist) for the rPTK of interest. The ligand may be an endogenous polypeptide, or a synthetic molecule, such as an inorganic or organic molecule. Usually, the ligand is a polypeptide. This assay is useful for screening molecules which activate
25 (or antagonize activation) of the tyrosine kinase receptor of interest. Thus, the assay can be used for developing therapeutically effective molecules.

- Where the ligand is an agonist, the molecule can comprise the native growth factor e.g., heregulin (HRG), insulin, insulin-like growth factors
30 I and II (IGF-I and IGF-II), epidermal growth factor (EGF), interleukins (e.g., IL-8), macrophage colony-stimulating factor (M-CSF), erythropoietin (EPO), platelet-derived growth factor (PDGF), transforming growth factors alpha and beta (TGF- α and TGF- β), hepatocyte growth factor (HGF), fibroblast growth factor (FGF) and nerve growth factor (NGF). Many of these
35 growth factors are available commercially. Alternatively, the growth factor can be made by peptide synthesis or recombinant techniques which are described herein. Synthetic small molecule agonists can similarly be

generated by those skilled in the art using conventional chemical synthesis techniques.

Where the ligand is present in a biological fluid, the analyte can be prepared using techniques which are well known in the art. Body fluid
5 such as blood or amniotic fluid may be used directly, however concentration may be required. If the analyte to be tested comprises a particular tissue, the cells thereof can be grown in cell culture and the supernatant can be tested for secreted ligand.

Often, the ligand is diluted in an aqueous diluent (such as cell
10 culture media) so that a standard curve can be generated. However, the ligand may be present in a cell or a cell component (e.g., the cell membrane). In particular, it has been found that the assay can be used to detect the presence of a ligand in the cell membrane of a selected cell line. This is clearly useful for discovering a novel endogenous ligand for
15 a known rPTK.

The ligand composition is added to each well which contains the adhering cells using a pipette, for example. At least one control well (e.g. to which the aqueous diluent for the ligand is added) is included in the assay.

20 The adhering cells are usually stimulated for a sufficient period of time to optimize the signal, but not too long such that the signal decreases as a consequence of dephosphorylation of the rPTK by endogenous phosphatases. A suitable stimulation period is between about 10 to 60 minutes, preferably about 30 minutes at a physiologically optimal
25 temperature for the cells (usually about 37°C).

Following activation, well supernatants are decanted and the plates can then be lightly tamped with an absorbent substrate to remove excess supernatant.

The assay can be used to detect antagonist ligands for the rPTK of
30 interest. Antagonists generally fall into two categories (a) ones which bind to the rPTK and thereby block binding and/or activation of the rPTK by an agonist thereto (the antagonist may bind to the ECD, but this is not necessarily the case) and (b) those which bind to the agonist and thus prevent activation of the rPTK by the agonist.

35 In order to detect antagonist molecules from category (a) above, the cells are exposed to the suspected antagonist ligand substantially as mentioned above. Following exposure to the antagonist, the well supernatants are decanted and the plates are lightly tamped. Then, a known

agonist (e.g., the endogenous growth factor) is added to the washed cells essentially as discussed in the preceding paragraphs, following which, the well supernatants are decanted and plates are lightly tamped. Alternatively, a composition comprising both the antagonist and agonist can be added to the adhering cells substantially as discussed above. Ability of the suspected antagonist to block binding and/or activation of the rPTK can subsequently be measured by ELISA as discussed below.

To detect antagonist molecules from category (b) above, a known agonist is pre-incubated with the suspected antagonist prior to the KIRA stage of the assay. This incubation is carried out for a sufficient period of time to enable a complex of the antagonist-agonist to form; from 30 min. to 12 hours, for example. This complex is then subjected to the assay with the non-complexed agonist and antagonist used as controls.

Following exposure to the agonist (and optionally the antagonist) ligand, the cells are lysed, as discussed below.

E. Solubilizing the cells

In this step of the assay, the cells are lysed so as to solubilize the rPTK such that it remains activated (i.e., the tyrosine residues remain phosphorylated) for the ELISA stage of the assay. Thus, the cells are lysed using a lysis buffer as described above which serves to solubilize the rPTK or receptor construct, yet does not dephosphorylate or denature the rPTK.

Where microtiter plates are used as mentioned above, about 75 to 200 μ l of lysis buffer is added to each well. The plates can then be agitated gently using a plate shaker (e.g., such as that sold by Bellco Instruments, Vineland, NJ) for about 1 to 2 hours. Shaking can be carried out at room temperature.

2. Enzyme-Linked Immunosorbent Assay - ELISA

The second stage of the assay involves a sandwich ELISA performed in the second assay plate. In order to carry out the ELISA, a capture agent is prepared.

A. Preparation of the capture agent

As mentioned above, the capture agent often comprises a polyclonal antibody (usually an affinity purified polyclonal antibody) or monoclonal antibody. Other capture agents are envisaged and are discussed in the definitions section above. The capture agent either binds specifically to the kinase receptor, or to the flag polypeptide (i.e. the antigen).

Polyclonal antibodies to the antigen (either the receptor or the flag polypeptide) generally are raised in animals by multiple subcutaneous (sc)

or intraperitoneal (ip) injections of the antigen or an antigenic fragment thereof (often the ECD of the rPTK) and an adjuvant. It may be useful to conjugate the antigen or a fragment containing the target amino acid sequence to a protein that is immunogenic in the species to be immunized
5 (e.g., keyhole limpet hemocyanin), using a bifunctional or derivatizing agent.

The route and schedule for administration of immunogen to the host animal or cultured antibody-producing cells therefrom are generally in keeping with established and conventional techniques for antibody
10 stimulation and production. While mice are frequently employed as the test model, it is contemplated that any mammalian subject including human subjects or antibody-producing cells obtained therefrom can be manipulated according to the processes of this invention to serve as the basis for production of mammalian, including human, hybrid cell lines.

15 Animals are typically immunized against the immunogenic conjugates or derivatives by combining 1 mg or 1 μ g of conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of conjugate in Freund's
20 complete adjuvant (or other suitable adjuvant) by subcutaneous injection at multiple sites. 7 to 14 days later animals are bled and the serum is assayed for anti-antigen titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different
25 cross-linking agent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

After immunization, monoclonal antibodies can be prepared by recovering immune cells (typically spleen cells or lymphocytes from lymph
30 node tissue) from immunized animals and immortalizing the cells in conventional fashion, e.g., by fusion with myeloma cells or by Epstein-Barr (EB)-virus transformation and screening for clones producing the desired antibody. The hybridoma technique described originally by Kohler and Milstein, Eur. J. Immunol. 6:511 (1976), and also described by Hammerling
35 et al., In: Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, N.Y., pp. 563-681 (1981) has been widely applied to produce hybrid cell lines that secrete high levels of monoclonal antibodies against many specific antigens.

It is possible to fuse cells of one species with another. However, it is preferable that the source of the immunized antibody producing cells and the myeloma be from the same species.

The hybrid cell lines can be maintained in culture in cell culture media. The cell lines of this invention can be selected and/or maintained in a composition comprising the continuous cell line in hypoxanthine-aminopterin-thymidine (HAT) medium. In fact, once the hybridoma cell line is established, it can be maintained on a variety of nutritionally adequate media. Moreover, the hybrid cell lines can be stored and preserved in any number of conventional ways, including freezing and storage under liquid nitrogen. Frozen cell lines can be revived and cultured indefinitely with resumed synthesis and secretion of monoclonal antibody.

The secreted antibody is recovered from tissue culture supernatant by conventional methods such as precipitation, ion exchange chromatography, affinity chromatography, or the like. The antibodies described herein are also recovered from hybridoma cell cultures by conventional methods for purification of IgG or IgM, as the case may be, that heretofore have been used to purify these immunoglobulins from pooled plasma, e.g., ethanol or polyethylene glycol precipitation procedures. The purified antibodies are then sterile filtered. Where the antibody is a polyclonal antibody, it is generally affinity purified using an affinity column generated from the antigen of interest so as to provide a substantially specific capture antibody. Affinity chromatography is usually preceded by other purification techniques, such as liquid chromatography.

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated via the techniques described in McCafferty et al., *Nature*, 348:552-554 (1990), using the flag polypeptide, rPTK, or a fragment thereof, to select for a suitable antibody or antibody fragment. Clackson et al., *Nature*, 352:624-628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Mark et al., *Bio/Technol.* 10:779-783 [1992]), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids Res.*, 21:2265-2266 [1993]). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for

isolation of "monoclonal" antibodies which are encompassed by the present invention.

DNA encoding the monoclonal antibodies of the invention is readily isolated and sequenced using conventional procedures (e.g., by using
5 oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary
10 (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences, Morrison et al., Proc.
15 Nat. Acad. Sci. 81, 6851 (1984), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In that manner, "chimeric" or "hybrid" antibodies are prepared that have the binding specificity of an anti-rPTK or anti-flag polypeptide monoclonal antibody herein. Thus, the antibody may
20 be made by recombinant DNA methods (Cabilly et al., U.S. Pat. No. 4,816,567).

Binding of the capture agent is not affected by the presence or absence of a ligand bound to the receptor and the capture agent does not sterically block access to the phosphorylated tyrosine(s) by the anti-
25 phosphotyrosine antibody. Furthermore, the capture agent does not, of course, activate the receptor of interest. In order to screen for an antibody having these characteristics, the procedure outlined in Figure 3 can be carried out.

First, once the capture agent (e.g. an antibody or streptavidin) has
30 been chosen, binding to either the receptor or the flag polypeptide (where a receptor construct is to be used in the assay) is confirmed. This can be determined by flow cytometric analysis, immuno-precipitation or antigen-coat ELISA, for example. Flow cytometric analysis has been described above. Immunoprecipitation usually involves lysing the cells (having the
35 receptor or receptor construct) in non-ionic detergent (e.g. 0.5% Triton X-100) in a suitable buffer (e.g. PBS) and the cell lysates thus obtained are then incubated with the potential anti-receptor or anti-flag polypeptide capture agent. The immune complexes are precipitated with

either (a) anti-capture agent antibodies in the presence of polyethylene glycol (PEG) which enhances precipitation of the immune complex or with (b) insoluble (e.g. agarose bound) protein A or protein G. The immunoprecipitated material is then analyzed by polyacrylamide gel electrophoresis (PAGE). For antigen-coat ELISA, ELISA wells are coated overnight with either the purified receptor, purified flag polypeptide or purified receptor construct. The coated wells are then exposed to the potential capture agent and screened with HRPO-conjugated species specific anti-capture agent antibody.

The ability of the capture agent to bind to the receptor or flag polypeptide in the presence of a ligand to the receptor is also confirmed. This can be analyzed by incubating the receptor or receptor construct with a known ligand for the receptor (e.g. the endogenous growth factor or an agonist antibody thereto). Flow cytometric analysis, immunoprecipitation or antigen-coat ELISA can then be performed substantially as described above to investigate binding of the capture agent.

Assuming the capture agent is suitable as determined by the preceding two steps, it is then shown that the capture agent does not induce receptor activation (i.e. autophosphorylation) either before or after cell lysis. Thus, the cell-bound receptor or receptor construct is exposed to either the potential capture agent or a negative control (e.g. a control antibody which does not activate the receptor). Following cell lysis, the receptor or receptor construct can be subjected to Western blot analysis using labeled anti-phosphotyrosine antibodies. See, e.g., Glenney et al., Journal of Immunological Methods 109:277-285 (1988); Kamps, Methods in Enzymology 201:101-110 (1991); Kozma et al., Methods in Enzymology 201:28-43 (1991); or Holmes et al., Science 256:1205-10 (1992). To establish whether the capture agent induces receptor activation following cell lysis, a trial run of the KIRA ELISA (with both the capture agent and a negative control as discussed above) can be performed.

Finally, the ability of an anti-phosphotyrosine antibody (e.g. biotinylated anti-phosphotyrosine antibody) to bind the activated receptor in the presence of the potential capture agent is confirmed by a trial run in the KIRA ELISA disclosed herein.

Assuming the capture agent meets all the criteria specified above, it has good potential for use in the KIRA ELISA.

Once a suitable capture agent has been prepared, the second solid phase is coated therewith. Between about 0.1 to 10 $\mu\text{g/ml}$ of capture agent

can be added to each well of the second assay plate using a pipette, for example. The capture agent is often provided in a buffer at a high pH (e.g., between about 7.5 to 9.6) so that it has an increased overall charge and therefore displays enhanced binding to the second assay plate. Usually, 5 the capture agent will be incubated in the wells for between about 8 to 72 hours to enable a sufficient coating of the capture agent to form on the inside walls of the wells. This incubation is generally carried out at low temperatures (e.g., between about 3-8°C) to avoid or reduce degradation of the capture agent.

10 Following incubation, the wells of the plate are decanted and tamped lightly with an absorbent substrate. Non-specific binding is then blocked. In order to achieve this, a block buffer, is added to the wells. For example, a block buffer containing bovine serum albumin (BSA) such as that sold by Intergen Company, Purchase, NY, is suitable. It has been found that 15 addition of between about 100 to 200 μ l of block buffer to each well followed by gentle agitation at room temperature for between about 1-2 hours is sufficient to block non-specific binding. It is also possible to add the block buffer directly to the cell lysate obtained in the previous step rather than to the second assay plate.

20 Following this, the capture agent-coated plates are washed several times (usually between about 3-8 times) with a wash buffer. The wash buffer can comprise phosphate buffered saline (PBS) at pH 7.0 to 7.5, for example. However, other wash buffers are available which can also be used. Conveniently, an automated plate washer, such as the ScanWasher 300 25 (Skatron Instruments, Inc., Sterling, VA) can be used for this, and other, washing steps of the assay.

B. Measuring tyrosine phosphorylation

The activated, solubilized rPTK (or receptor construct) is then added to the wells having the capture agent adhering thereto. As a general 30 proposition, about 80% of cell lysate obtained as mentioned under Section 1E above can be added to each well (i.e., about 60 to 160 μ l depending on the original volume of the wells). The lysate is incubated with the capture agent for an adequate period of time to enable the rPTK to be captured in the wells, e.g., from 1 to 3 hours. Incubation can be carried 35 out at room temperature.

Unbound cell lysate is then removed by washing with wash buffer. Following this washing step, an amount of the anti-phosphotyrosine antibody which is equal to, or less than, the amount of block buffer added

previously, is added to each well. For example, about 50 to 200 μ l of an anti-phosphotyrosine antibody preparation having between about 0.3 to 0.5 μ g/ml of antibody in a suitable buffer (e.g., PBS with a detergent such as those included in the lysis buffer) is added to the well. This is followed
5 by a washing step to remove unbound anti-phosphotyrosine antibody.

Tyrosine phosphorylation is then quantified by the amount of anti-phosphotyrosine antibody binding to the second solid phase. Many systems for detecting the presence of an antibody are available to those skilled in the art. Some examples follow.

10 Generally, the anti-phosphotyrosine antibody will be labelled either directly or indirectly with a detectable label. Numerous labels are available which can be generally grouped into the following categories:

(a) Radioisotopes, such as ^{35}S , ^{14}C , ^{125}I , ^3H , and ^{131}I . The antibody can be labeled with the radioisotope using the techniques described in
15 Current Protocols in Immunology, supra, for example and radioactivity can be measured using scintillation counting.

(b) Fluorescent labels such as rare earth chelates (europium chelates) or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, Lissamine, phycoerythrin and Texas Red are available.
20 The fluorescent labels can be conjugated to the antibody using the techniques disclosed in Current Protocols in Immunology, supra, for example. Fluorescence can be quantified using a fluorimeter (Dynatech).

(c) Various enzyme-substrate labels are available and U.S. Patent No. 4,275,149 provides a review of some of these. The enzyme generally
25 catalyses a chemical alteration of the chromogenic substrate which can be measured using various techniques. For example, the enzyme may catalyze a color change in a substrate, which can be measured spectrophotometrically. Alternatively, the enzyme may alter the fluorescence or chemiluminescence of the substrate. Techniques for quantifying a change in fluorescence are
30 described above. The chemiluminescent substrate becomes electronically excited by a chemical reaction and may then emit light which can be measured (using a Dynatech ML3000 chemiluminometer, for example) or donates energy to a fluorescent acceptor. Examples of enzymatic labels include luciferases (e.g., firefly luciferase and bacterial luciferase; U.S. Patent
35 No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, malate dehydrogenase, urease, peroxidase such as horseradish peroxidase (HRPO), alkaline phosphatase, β -galactosidase, glucoamylase, lysozyme, saccharide oxidases (e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate

dehydrogenase), heterocyclic oxidases (such as uricase and xanthine oxidase), lactoperoxidase, microperoxidase, and the like. Techniques for conjugating enzymes to antibodies are described in O'Sullivan et al., Methods for the Preparation of Enzyme-Antibody Conjugates for use in Enzyme
5 Immunoassay, in Methods in Enzym. (ed J. Langone & H. Van Vunakis), Academic press, New York, 73: 147-166 (1981) and Current Protocols in Immunology, supra.

Examples of enzyme-substrate combinations include, for example:

(i) Horseradish peroxidase (HRPO) with hydrogen peroxidase as a
10 substrate, wherein the hydrogen peroxidase oxidizes a dye precursor (e.g. orthophenylene diamine [OPD] or '3,3',5,5'-tetramethyl benzidine hydrochloride [TMB]).

(ii) alkaline phosphatase (AP) with para-Nitrophenyl phosphate as chromogenic substrate.

15 (iii) β -D-galactosidase (β -D-Gal) with a chromogenic substrate (e.g. p-nitrophenyl- β -D-galactosidase) or fluorogenic substrate 4-methylumbelliferyl- β -D-galactosidase.

Numerous other enzyme-substrate combinations are available to those skilled in the art. For a general review of these, see U.S. Patent Nos.
20 4,275,149 and 4,318,980.

Sometimes, the label is indirectly conjugated with the antibody. The skilled artisan will be aware of various techniques for achieving this. For example, the antibody can be conjugated with biotin and any of the three broad categories of labels mentioned above can be conjugated with avidin,
25 or vice versa. Biotin binds selectively to avidin and thus, the label can be conjugated with the antibody in this indirect manner. See, Current Protocols in Immunology, supra, for a review of techniques involving biotin-avidin conjugation. Alternatively, to achieve indirect conjugation of the label with the antibody, the antibody is conjugated with a small
30 hapten (e.g. digoxin) and one of the different types of labels mentioned above is conjugated with an anti-hapten antibody (e.g. anti-digoxin antibody). Thus, indirect conjugation of the label with the antibody can be achieved.

In another embodiment of the invention, the anti-phosphotyrosine
35 antibody need not be labeled, and the presence thereof can be detected using a labeled anti-antiphosphotyrosine antibody (e.g. anti-mouse anti-phosphotyrosine antibody conjugated with HRPO).

In the preferred embodiment, the anti-phosphotyrosine antibody is labeled with an enzymatic label which catalyzes a color change of a substrate (such as tetramethyl benzimidine [TMB], or orthaphenylene diamine [OPD]). Thus, the use of radioactive materials is avoided. A color change of the reagent can be determined spectrophotometrically at a suitable wavelength (e.g. 450nm for TMB and 490nm for OPD, with a reference wavelength of 650 nm).

3. Intracellular Kinase Activity

The assay described herein is also useful for measuring phosphorylation and/or activation of intracellular kinases (e.g. cytoplasmic tyrosine kinases and/or cytoplasmic serine-threonine kinases). Phosphorylation of these molecules can occur as a consequence of trans-phosphorylation of the intracellular kinase by a kinase receptor or "receptor complex" (which comprises one or more kinase receptors residing in a cell membrane). Examples of intracellular tyrosine kinases include insulin receptor substrate I (IRS-1), Shc, Ras and GRB2, for example. Antibodies to human Shc, human Ras and GRB2 can be obtained commercially from UBI, NY, which can be used as capture agents for these tyrosine kinases. Examples of intracellular serine-threonine kinases include MEK and MAPK.

In order to measure phosphorylation of these kinases, the procedure is essentially as described above except that a chimera of the intracellular kinase and the flag polypeptide is normally formed (i.e. a "kinase construct"). Alternatively, the cell has an endogenous intracellular kinase or is transformed with nucleic acid encoding an intracellular kinase of interest. Generally, a eukaryotic cell will be transformed with nucleic acid encoding a kinase construct. Upon expression of the nucleic acid, the kinase or kinase construct will reside intracellularly (i.e. in the cytoplasm). The cells comprising the kinase or kinase construct are subjected to the KIRA as discussed above. Exposure to the agonist may result in trans-phosphorylation of the intracellular kinase which can be quantified in the ELISA as elaborated above. The capture agent in the ELISA binds to either the intracellular kinase or to the flag polypeptide.

4. Serine-Threonine Kinase Activity

This assay is further useful for measuring phosphorylation and/or activation of serine-threonine kinases. The term "serine-threonine kinase" refers to a kinase which phosphorylates a substrate which has at least one

phosphate accepting alcohol group. The serine-threonine kinase is usually a "receptor" insofar as it has a ligand-binding ECD, TM domain and ICD. The ICD usually comprises a catalytic kinase domain and generally has one or more phosphate accepting serine and/or threonine residues. Examples of intracellular serine-threonine kinases include MEK and MAPK. See section 3 above for a discussion as to measuring phosphorylation of intracellular serine-threonine kinases. Examples of serine-threonine kinase receptors include daf-1, activin type II receptor (ActR-II), activin type IIB receptor (ActR-IIB), TGF- β type II receptor (T β R-II), activin receptor-like kinase (ALK) -1, -2, -3, -4 and TGF- β type I receptor (T β R-1)/ALK-5. See ten Dijke et al., supra. The serine-threonine kinase assay is essentially the same as described above for tyrosine kinases, except that phosphorylation is quantified using anti-phosphoserine and/or anti-phosphothreonine antibodies. Anti-phosphoserine and anti-phosphothreonine monoclonal antibodies can be purchased from Sigma Immuno Chemicals, St Louis, MO, for example.

5. Phosphatase Activity

Phosphatase activity can similarly be measured using the assay described herein. Phosphatase enzymes are able to dephosphorylate phosphorylated tyrosine, serine and/or threonine residues (i.e. liberate inorganic phosphate from phosphoric esters of such amino acid residues). Generally the phosphatase enzyme is specific for either tyrosine residues or serine-threonine residues but sometimes can dephosphorylate tyrosine, serine and threonine residues. Sometimes "endogenous" phosphatase activity is measured and this refers to the activity of phosphatase enzyme(s) which exist in nature in a selected cell.

In order to quantify endogenous phosphatase activity, cells possessing at least one phosphatase are stimulated in the presence and absence of one or more phosphatase inhibitors. Examples of protein tyrosine phosphatase (PTPase) inhibitors include sodium orthovanadate and sodium molybdate (Sigma Chemical Co., St. Louis, MO). ICN Biochemicals supply okadaic acid which is a serine-threonine phosphatase inhibitor. As a general proposition, between about 1-10 μ M phosphatase inhibitor can be added to each well of the assay plate. In all other respects, the assay is performed essentially as discussed above. Thus, the ability of endogenous phosphatases to dephosphorylate a kinase in the selected cell can be quantified.

In the preferred embodiment, a phosphatase enzyme of interest can be studied. Examples of protein tyrosine phosphatases (PTPases) include PTP1B, PTPMEG, PTP1c, Yop51, VHL, cdc25, CD45, HLAR, PTP18, HPTP α and DPTP10D. See Zhang and Dixon, Adv. Enzym. 68: 1-36 (1994). Examples of protein serine-threonine phosphatases include PP1, PP2A, PP2B and PP2C. See Meth. Enzym., ed Hunter & Sefton, Academic press, New York, 201:389-398 (1991). These proteins can be purchased commercially or made using the recombinant techniques described herein. To measure phosphatase activity, the KIRA ELISA can be performed essentially as described above with the following modifications. Following capture of the kinase or kinase construct (e.g. receptor construct) to the second solid phase and the washing step (to remove unbound cell lysate), the phosphatase of interest is added to the wells of the second assay plate and incubated with the adhering kinase or kinase construct. For example, between about 50-200 μ l of the phosphatase in a suitable dilution buffer (see Meth. Enzym., ed Hunter & Sefton, Academic press, New York, 201:416-440 [1991]) can be added to each well. This is generally followed by gentle agitation at room temperature (or 37°C) for between about 30 min to 2 hours to allow the phosphatase to dephosphorylate the kinase. Following washing to remove the phosphatase, the decreased degree of phosphorylation of the kinase relative to the control (i.e. no phosphatase added) is quantified by ELISA as described earlier herein.

6. Kits

As a matter of convenience, the reagents can be provided in a kit, i.e., a packaged combination of reagents, for combination with the analyte in assaying the ability of the analyte to activate or prevent activation of a rPTK of interest. The components of the kit will be provided in predetermined ratios. Thus, a kit will comprise the specific second solid phase for the assay as well as the anti-flag polypeptide capture agent either packaged separately or captured to the second solid phase (e.g. a microtiter plate). Usually, other reagents, such as the anti-phosphotyrosine antibody labelled directly or indirectly with an enzymatic label will also be provided in the kit. Where the detectable label is an enzyme, the kit will include substrates and cofactors required by the enzyme (e.g. a substrate precursor which provides the detectable chromophore or fluorophore). In addition, other additives may be included such as stabilizers, buffers (e.g. a block buffer and a lysis buffer) and the like. Conveniently, the kit can also supply the homogeneous population

of cells which have been transformed with the receptor construct. The relative amounts of the various reagents may be varied widely to provide for concentrations in solution of the reagents which substantially optimize the sensitivity of the assay. Particularly, the reagents may be provided
5 as dry powders, usually lyophilized, including excipients which on dissolution will provide a reagent solution having the appropriate concentration. The kit also suitably includes instructions for carrying out the KIRA ELISA.

7. Uses for the Assay

10 This application provides two assays which are useful for reliable, sensitive and quantitative detection of kinase activation. The first assay can be used where a kinase receptor-specific capture antibody having the desired characteristics herein described is available or has been prepared. The second assay is a generic assay which enables activation of any kinase
15 receptor to be measured via the use of a flag polypeptide and a capture agent which binds specificity thereto.

These assays are useful for identifying novel agonists/antagonists for a selected kinase receptor. Also, the assay provides a means for studying ligand-receptor interactions (i.e., mechanism studies). Also the
20 presence of an endogenous receptor in a selected cell line can be quantified using the assay. The assays are further useful for identifying the presence of a ligand for a selected kinase receptor in a biological sample and, e.g., establishing whether a growth factor has been isolated following a purification procedure. It is desirable to have an assay for
25 measuring the ability of these growth factors to activate their respective receptors.

The assay also has clinical applications for detecting the presence of a ligand for a selected rPTK (e.g. the insulin receptor) in a biological sample taken from a human and thus patients having elevated or depressed
30 levels of the ligand can be identified. This is particularly desirable where elevated or depressed levels of the ligand cause a pathological condition. Accordingly, candidates for administration of the selected ligand (e.g. insulin) can be identified through this diagnostic method. It is possible, using the assay disclosed herein, to assay the pK of agonists
35 or antagonists administered to a patient. This assay also facilitates the detection of shed receptor in a biological sample.

The assay is also useful to quantify phosphatase activity of endogenous phosphatases or, in the preferred embodiment, a phosphatase of

interest. This can be used for screening phosphatase inhibitors, for example.

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

All publications, patents and patent applications cited herein, whether supra or infra, are hereby incorporated by reference in their entirety.

10

EXAMPLE 1

KIRA ELISA of the HER2 Receptor

The assay system described in this example was developed to measure the extent of autophosphorylation as a result of the interactions between the HER2 receptor and its specific activator, heregulin (HRG). The overexpression of p185^{HER2} has been correlated with poor clinical outcome in a number of epithelial-derived cancers. Heregulin and its rodent homologue, neu differentiation factor (NDF), were originally purified based on their ability to stimulate the autophosphorylation of a 185 kDa protein in the breast carcinoma cell lines MCF-7 and MDA-453, respectively. In this embodiment of the invention, the cell line expressing the tyrosine kinase receptor DNA (either endogenous or transformed) is adherent and there is an antibody (e.g. monoclonal or affinity purified polyclonal) capable of specifically binding the receptor such that it neither stimulates autophosphorylation in the absence of ligand nor suffers impaired binding due to the presence of bound ligand. Standard curve preparations and many samples may easily be run simultaneously in replicate and at several dilutions using this assay, readily allowing quantitation of ligand activity in a large number of unknown samples.

(i) Capture agent preparation

30 Polyclonal anti-HER2 antibody was isolated from pooled immune sera from New Zealand White rabbits immunized with the extracellular domain of the HER2 molecule (Fendly et al., Journal of Biological Response Modifiers 9:449-455 [1990]). The rHER2 ECD specific antibodies were affinity purified using an FPLC (Pharmacia Biotech, Inc, Piscataway, NJ) with an affinity column generated from rHER2 ECD conjugated to Avidgel F (Bioprobe International, Inc, Tustin, CA). The resulting purified antibody stock was

0.829 mg/ml in phosphate buffered saline (PBS), pH 7.4, and was stored as 0.5 ml aliquots at -20°C.

(ii) Anti-phosphotyrosine antibody preparation

Monoclonal anti-phosphotyrosine, clone 4G10, was purchased from Upstate Biologicals, Inc (UBI, Lake Placid, NY) and biotinylated using long-arm biotin-N-hydroxysuccinamide (Biotin-X-NHS, Research Organics, Cleveland, OH).

(iii) Ligand

The recombinant truncated form of β heregulin (MW= 7.88 Kd) corresponding to residues 177-244 (HRG β ₁₇₇₋₂₄₄) was produced in *E. coli* and purified to homogeneity as described in Holmes et al., *Science*, 256: 1205-1210 (1992) and was stored at 4°C as an 89.7 μ M stock solution in 50 mM Tris/HCl, pH 7.5.

(iv) Adherent Cells

MCF-7 (ATCC-HTB 22), an adherent cell line isolated from a human breast adenocarcinoma, was obtained from American Type Culture Collection (ATCC, Rockville, MD). MCF-7 cells have been shown to produce measurable levels of surface p185^{HER2} by both FACS and ELISA analysis. The cells were maintained in 150 cm² tissue culture flasks (Corning Inc, Corning, NY) and utilized when at cell densities of 60 % to 75 % confluency. For the assay, 2 x 10⁵ cells were seeded per well in flat-bottom microtiter plates (Falcon 3072, Becton Dickinson Labware, Lincoln Park, NJ) cultured overnight at 37°C in 5 % CO₂. Cells were grown in F12/DMEM 50:50 Gibco as a custom formulation (Gibco/BRL, Life Technologies, Grand Island, NY). The medium was supplemented with 10 % FBS (Hyclone, Logan, Utah), 25 mM HEPES (Gibco) and 2 mM L-glutamine (Gibco).

(v) KIRA ELISA

MCF-7 cells (2 x 10⁵) in 100 μ l media were added to each well in a flat-bottom-96 well culture plate and cultured overnight at 37°C in 5% CO₂. The following morning the well supernatants were decanted, and the plates were lightly tamped on a paper towel. 50 μ l of media containing either experimental samples or the recombinant HRG β ₁₇₇₋₂₄₄ standards (3000, 1000, 333, 111, 37, 12, 4, and 0 pM) was then added to each well. The cells were stimulated at 37°C for 30 min., the well supernatants were decanted, and the plates were once again lightly tamped on a paper towel. To lyse the cells and solubilize the receptors, 100 μ l of lysis buffer was added to each well. Lysis buffer consisted of 150 mM NaCl containing 50 mM HEPES (Gibco), 0.5 % Triton-X 100 (Gibco), 0.01 % thimerosal, 30 KIU/ml aprotinin

(ICN Biochemicals, Aurora, OH), 1mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF; ICN Biochemicals), 50 μ M leupeptin (ICN Biochemicals), and 2 mM sodium orthovanadate (Na_3VO_4 , Sigma Chemical Co, St. Louis, MO), pH 7.5. The plate was then agitated gently on a plate shaker
5 (Bellco Instruments, Vineland, NJ) for 60 min. at room temperature.

While the cells were being solubilized, an ELISA microtiter plate (Nunc Maxisorp, Inter Med, Denmark) coated overnight at 4°C with the affinity-purified polyclonal anti-HER2 ECD (1.0 μ g/ml in 50 mM carbonate buffer, pH 9.6, 100 μ l/well) was decanted, tamped on a paper towel and
10 blocked with 150 μ l/well of Block Buffer [PBS containing 0.5 % BSA (Intergen Company, Purchase, NY) and 0.01 % thimerosal] for 60 min. at room temperature with gentle agitation. After 60 minutes, the anti-HER2 ECD coated plate was washed 6 times with wash buffer (PBS containing 0.05 % Tween-20 and 0.01 % thimerosal) using an automated plate washer (ScanWasher
15 300, Skatron Instruments, Inc, Sterling, VA).

The lysate containing solubilized p185^{HER2} from the cell-culture microtiter well was transferred (85 μ l/well) to anti-rHER2 ECD coated and blocked ELISA well and was incubated for 2 h at room temperature with gentle agitation. The unbound receptor was removed by washing with wash
20 buffer and 100 μ l of biotinylated 4G10 (anti-phosphotyrosine) diluted 1:2000 in dilution buffer (PBS containing 0.5 % BSA, 0.05 % Tween-20, 5 mM EDTA, and 0.01 % thimerosal), i.e. 400pg/ml, was added to each well. After incubation for 2 h at room temperature the plate was washed and 100 μ l of HRPO-conjugated streptavidin (Zymed Laboratories, S. San Francisco, CA)
25 diluted 1:10000 in dilution buffer was added to each well. The plate was incubated for 30 minutes at room temperature with gentle agitation. The free avidin-conjugate was washed away and 100 μ l freshly prepared substrate solution (tetramethyl benzidine [TMB]; 2-component substrate kit; Kirkegaard and Perry, Gaithersburg, MD) was added to each well. The
30 reaction was allowed to proceed for 10 minutes, after which the color development was stopped by the addition of 100 μ l/well 1.0 M H_3PO_4 . The absorbance at 450 nm was read with a reference wavelength of 650 nm ($\text{ABS}_{450/650}$), using a vmax plate reader (Molecular Devices, Palo Alto, CA) controlled with a Macintosh Centris 650 (Apple Computers, Cupertino, CA)
35 and DeltaSoft software (BioMetallics, Inc, Princeton, NJ).

The standard curve shown in Figure 7 was generated by stimulating MCF-7 cells with 3000, 1000, 333, 111, 37, 12, 4, or 0 pM HRG β _{1,177-244} and presented as pM HRG β _{1,177-244} vs. mean $\text{ABS}_{450/650} \pm \text{sd}$ using the DeltaSoft

program. Sample concentrations were obtained by interpolation of their absorbance on the standard curve and are expressed in terms of pM HRG β _{1,177-244} activity.

When the data were fitted to a 4-parameter nonlinear least squares equation, they resulted in a correlation coefficient of 0.9998. For the data shown in Figure 7, the EC₅₀ of receptor activation by HRG β _{1,177-244} was 373 pM. To demonstrate the highly reproducible nature of the p185^{HER2} KIRA ELISA, seven standard curves were generated over the period of one month and the EC₅₀'s are averaged. This gives an EC₅₀ave for HRG β _{1,177-244} of 360 \pm 40 pM (average \pm SD).

(vi) Intra- and inter-assay precision and assay specificity

The intra-assay variability was determined by performing the p185^{HER2} KIRA ELISA on three separate days. For each test, the standard curve is run in triplicate. Controls with HRG β _{1,177-244} corresponding to high (1000 pM), mid (200 pM) and low (40 pM) were assayed in 24 replicates. The ABS_{450/650} of the individual test samples were converted to pM HRG β _{1,177-244} activity and the 24 converted values for each test concentration were averaged. The data are expressed as averaged value and % coefficient of variation (%cv; [(intra-assay standard deviation/intra-assay averaged calculated value) \times 100]. See Table 1A below.

Table 1

Intra- and Inter-assay Variation

A. Intra-assay Precision (n=24 per test)

	High Value ^a		Mid Value		Low Value	
	Average Value (pM)	% cv ^b	Average Value (pM)	% cv	Average Value (pM)	% cv
Test #1	1256	19.5%	209	10.8%	33	12.3%
Test #2	1078	10.0%	196	5.1%	38	7.5%
Test #3	999	14.3%	196	6.3%	35	11.3%

B. Inter-assay Precision (n=3)

Average Value (pM)	%cv ^C	Average Value (pM)	% cv	Average Value (pM)	% cv
1100	4.3%	200	6.3%	34	9.0%

- 5 ^a Expected high value: 1000 pM; mid value: 200 pM; low value: 40 pM
^b Intra-assay % cv determined as intra-assay sd/intra-assay average x 100
^c Inter-assay % cv determined as inter-assay sd/inter-assay average x 100

The intra-assay variability of the KIRA ELISA was within acceptable limits despite the fact that the assay actually consists of both bioassay
10 and ELISA components. The coefficients of variance (%) for the highest values were under 20% and for the mid and low values were at or under 10%.

The inter-assay variability was determined by averaging the values from upper-most three adjacent wells (of the 24 wells run) for a given sample concentration from each run. The three separate averages for each
15 test concentration were then averaged. The data were expressed as averaged value and %cv [(inter-assay standard deviation/inter-assay averaged value) x 100]. See Table 1B. above. The inter-assay variability of the KIRA ELISA was within acceptable limits.

In order to confirm the specificity of the assay, MCF-7 cells were
20 stimulated with either HRGB1₁₇₇₋₂₄₄ at 3000, 1000, 333, 111, 37, 12, 4 or 0 pM or insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), or insulin at 30000, 10000, 3333, 1111, 370, 120, 40 or 0 pM. The p185^{HER2} KIRA ELISA was then performed as described above. The results are depicted in Figure 8.

25 The p185^{HER2} KIRA ELISA was clearly specific for heregulin. While HRGB1₁₇₇₋₂₄₄ induced normal receptor stimulation and autophosphorylation, the closely related EGF gives only a slight stimulation (OD_{450/650} = 0.239) at the highest concentration tested (100 nM). Since EGF-R is produced in MCF-7 cells, this signal is likely due to EGF receptor transphosphorylation of
30 p185^{HER2}. Neither insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF) nor insulin have any detectable effect on the MCF-7 p185^{HER2} KIRA ELISA, the latter despite the fact that MCF-7 cells produce active insulin receptors.

The results presented in this example demonstrate that the KIRA ELISA
35 is a useful method for assaying ligand activation of a kinase receptor, e.g., heregulin activation of the p185^{HER2} receptor. Levels of receptor activation in terms of tyrosine phosphorylation are easily quantified and an EC₅₀ for a given ligand is readily determined. One potential use for this

assay would be to screen compounds for receptor agonist or antagonist activities. The potential throughput for this assay greatly surpasses that of Western blot analysis. Since the cell-culture portion of the assay is conducted in 96-well plates, many samples may be run in replicate at 5 different dilutions at one time in a one-day assay.

EXAMPLE 2

KIRA ELISA of the Rse Receptor

Mark et al., Journal of Biological Chemistry 269(14):10720-10728 (1994) describe isolation of the Rse receptor protein tyrosine kinase from 10 human and murine tissues. This Rse receptor with a carboxyl-terminal flag polypeptide (i.e. Rse.gD) was subjected to the KIRA ELISA described herein. The experimental procedure is outlined below.

(i) Capture agent preparation

Monoclonal anti-gD (clone 5B6) was produced against a peptide from 15 Herpes simplex virus glycoprotein D (Paborsky et al., Protein Engineering 3(6):547-553 [1990]). The purified stock preparation was adjusted to 3.0mg/ml in phosphate buffered saline (PBS), pH 7.4 and 1.0ml aliquots were stored at -20°C.

(ii) Anti-phosphotyrosine antibody preparation

20 Monoclonal anti-phosphotyrosine, clone 4G10, was purchased from Upstate Biologicals, Inc (UBI, Lake Placid, NY) and biotinylated using long-arm biotin-N-hydroxysuccinamide (Biotin-X-NHS, Research Organics, Cleveland, OH).

(iii) Ligand

25 Since the endogenous ligand for the Rse receptor was not available, an agonist antibody for the Rse receptor was prepared which forms the ligand for the KIRA ELISA described in this Example. To generate the agonist antibody, a Rse.IgG chimera was generated. Briefly, the coding sequence of the ECD of Rse was fused to that of the human IgG- γ 1 heavy 30 chain in a multi-step process. PCR was used to generate a fragment with a unique BstEII site 3' to the coding sequences of the Rse amino acid 428. The PCR product was joined to the human IgG- γ 1 heavy chain cDNA through a unique BstEII site in that construct (Mark et al., J. Cell. Biol., 267: 26166-26171 [1992]). The resulting construct (termed pRK.bpTK3.IgG.fusion) 35 contained the coding sequences for amino acids 375-428 of Rse joined to those encoding human IgG- γ 1 heavy chain. The remaining portion of the Rse

ECD (amino acids 1-374) was then added by linkage through the Bam HI site in pRK.bpTK3.IgG.fusion to yield pRK.Rse.IgG.

To generate stable cell populations expressing Rse.IgG, the cDNA encoding Rse.IgG was subcloned into the episomal CMV-driven expression
5 plasmid pCIS.EBON, a pRK5 derivative disclosed in Cachianes et al., Bio. Techniques, 15: 225-259 (1993). Human fetal kidney 293 cells (obtained from ATCC, 12301 Parklawn Drive, Rockville, MD, USA) were transfected by the calcium phosphate technique. Cell monolayers were incubated for four hours in the presence of the DNA precipitate, glycerol shocked, and cultured in
10 F12:DMEM (1:1) containing 2mM glutamine, 10% fetal bovine serum, penicillin and streptomycin. After 48 hours, populations were replated in media containing G418 to select for a stable population of cells. Conditioned media was collected from cells expressing Rse.IgG nucleic acid that have been cultured in serum-free media for 72 hours in the absence of G418.

15 Rse.IgG was purified by affinity chromatography on a protein A column using procedures as described by Chamow, S.M., et al., Biochemistry, 29:9885-9891 (1990) with the following minor modifications. Conditioned media collected from cells expressing the Rse.IgG was adjusted to 0.1 M citrate pH 6.0 and loaded directly onto a protein A column (Repligen). The
20 column was washed with 0.1 M citrate, pH 6.0, and was eluted with 3 M MgCl₂ with 10% glycerol. Fractions were pooled and desalted on a PD-10 column, dialyzed and concentrated against PBS. Protein concentrations were determined by an ELISA against human IgG (Fc). The protein was analyzed for purity by Coomassie staining of PAGE gels.

25 Polyclonal antibodies were generated in New Zealand white rabbits against the Rse.IgG formed as described above. 4µg of Rse.IgG in 100µL PBS was emulsified with 100µL Freund's adjuvant (complete adjuvant for the primary injection and incomplete adjuvant for all boosts). For the primary immunization and the first boost, the protein was injected directly into
30 the popliteal lymph nodes (Sigel et al., Methods Enzymol., 93, 3-12 [1983]). For subsequent boosts, the protein was injected into subcutaneous and intramuscular sites. 1.3 µg protein/kg body weight was injected every 3 weeks with bleeds taken 1 and 2 weeks following each boost. The polyclonal antisera generated was then precipitated in 50% ammonium
35 sulphate.

The resultant, purified polyclonal antisera is called "19B" herein. To confirm the ability of the 19B antisera to induce autophosphorylation of the Rse receptor, serum starved 3T3.gD.R11 cells (transformed with

nucleic acid encoding the Rse receptor with an amino terminal gD flag polypeptide [i.e. gD.Rse] using the techniques described in Mark et al., Journal of Biological Chemistry 269(14):10720-10728 [1994]) or NIH3T3 cells were exposed to pre-immune serum or 19B polyclonal antisera at a 1:200 dilution for 10 minutes. The gD.Rse protein was immunoprecipitated from extracts using the anti-gD monoclonal antibody 5B6. Proteins were fractionated on 7% SDS-PAGE under reducing conditions and transferred to nitrocellulose. Phosphorylation of Rse was detected with labelled anti-phosphotyrosine antibody. Treatment of the 3T3.gD.R11 cells with 19B antisera stimulated the phosphorylation of the 140kD gD.Rse protein. This increase was not observed in cells treated with pre-immune sera.

The purified 19B polyclonal antisera was stored at 4°C as an 2.8mg/ml stock solution in PBS, pH 7.5.

(iv) Preparation of Rse.gD nucleic acid

Synthetic double stranded oligonucleotides were used to reconstitute the coding sequence for the C-terminal 10 amino acids (880 - 890) of human Rse and add an additional 21 amino acids containing an epitope for the antibody 5B6 and a stop codon. The final sequence of the synthetic portion of the fusion gene was:

coding strand:

5'-TGCAGCAAGGGCTACTGCCACACTCGAGCTGCGCAGATGCTAGCCTCAAGATGGCT G
ATCCAAATCGATTCCGCGGCAAAGATCTTCCGGTCTGTAGAAGCT-3' (SEQ ID NO: 10)

noncoding (anti-sense) strand:

5'-AGCTTCTACAGGACCGGAAGATCTTTGCCGCGGAATCGATTTGGATCAGCCATCTT G
AGGCTAGCATCTGCGCAGCTCGAGTGTGGCAGTAGCCCTTGCTGCA-3' (SEQ ID NO: 11).

The synthetic DNA was ligated with the cDNA encoding amino acids 1-880 of human Rse at the PstI site beginning at nucleotide 2644 of the published human Rse cDNA sequence (Mark et al., Journal of Biological Chemistry 269(14):10720-10728 [1994]) and HindIII sites in the polylinker of the expression vector pSVI7.ID.LL (See Figure 16; SEQ ID NO: 9) to create the expression plasmid pSV.ID.Rse.gD. Briefly, the expression plasmid comprises a dicistronic primary transcript which contains sequence encoding DHFR bounded by 5' splice donor and 3' splice acceptor intron splice sites, followed by sequence that encodes the Rse.gD. The full length (non-spliced) message contains DHFR as the first open reading frame and therefore generates DHFR protein to allow selection of stable transformants.

(v) Cell transformation

dp12.CHO cells (EP 307,247 published 15 March 1989) were electroporated with 20 µgs of pSV.ID.Rse.gD which had been linearized at a unique NotI site in the plasmid backbone. The DNA was ethanol
5 precipitated after phenol/chloroform extraction and was resuspended in 20µl 1/10 Tris EDTA. Then, 10µg of DNA was incubated with 10⁷ CHO.dp12 cells in 1 ml of PBS on ice for 10 min. before electroporation at 400 volts and 330µf. Cells were returned to ice for 10 min. before being plated into non-selective medium. After 24 hours cells were fed nucleoside-free medium
10 to select for stable DHFR+ clones.

(vi) Selection of transformed cells for use in the KIRA ELISA

To identify a cell line that expresses Rse.gD nucleic acid, candidate clones were screened by fluorescence activated cell sorting (FACS) analysis using the polyclonal antiserum 19B generated as described above, which
15 recognizes epitopes in the extracellular domain of Rse. See Figure 5, step (b).

To confirm that clones that scored positive in the FACS assay express full-length Rse.gD nucleic acid, cell lysates were prepared (Lokker et al., EMBO J. 11:2503-2510 [1992]) and solubilized Rse.gD was immunoprecipitated
20 with the 19B antisera. The immunoprecipitated proteins were fractionated under reducing conditions using 7% PAGE, blotted onto nitrocellulose and then probed with the anti-gD 5B6 antibody which was detected with a horseradish peroxidase conjugated anti-mouse IgG antibody. See Figure 5, step (c). The ability of Rse.gD in cell clones to be activated to undergo
25 autophosphorylation in response to the 19B agonistic antibody was determined. Briefly, serum starved dp.CHO cells transformed with Rse.gD nucleic acid as described above were exposed to pre-immune or 19B antisera at a 1:200 dilution for 10 min. The Rse.gD protein was immunoprecipitated from extracts using the anti-gD 5B6 monoclonal antibody. Proteins were
30 fractionated on 7% SDS-PAGE under reducing conditions and transferred to nitrocellulose. Phosphorylation of Rse was detected with labelled antiphosphotyrosine antibody. See Figure 5, step (d).

(vii) Media

Cells were grown in F12/DMEM 50:50 (Gibco/BRL, Life Technologies,
35 Grand Island, NY). The media was supplemented with 10% diafiltered FBS (Hyclone, Logan, Utah), 25mM HEPES and 2mM L-glutamine.

(viii) KIRA ELISA

- Rse.gD transformed dp12.CHO cells (EP 307,247 published 15 March 1989) were seeded (5×10^4 per well) in the wells of a flat-bottom-96 well culture plate in 100 μ l media and cultured overnight at 37°C in 5% CO₂. The following morning the well supernatants were decanted, and the plates were lightly tamped on a paper towel. 100 μ l of media containing either experimental samples or 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200 or 0 diluted, anti-Rse agonist polyclonal antibody (19B pAb) was then added to each well. The cells were stimulated at 37°C for 30 min., the well supernatants were decanted, and the plates were once again lightly tamped on a paper towel. To lyse the cells and solubilize the receptors, 100 μ l of lysis buffer was added to each well. Lysis buffer consisted of 150 mM NaCl containing 50 mM HEPES (Gibco), 0.5 % Triton-X 100 (Gibco), 0.01 % thimerosal, 30 KIU/ml aprotinin (ICN Biochemicals, Aurora, OH), 1mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF; ICN Biochemicals), 50 μ M leupeptin (ICN Biochemicals), and 2 mM sodium orthovanadate (Na₃VO₄; Sigma Chemical Co, St. Louis, MO), pH 7.5. The plate was then agitated gently on a plate shaker (Bellco Instruments, Vineland, NJ) for 60 min. at room temperature.
- While the cells were being solubilized, an ELISA microtiter plate (Nunc Maxisorp, Inter Med, Denmark) coated overnight at 4°C with the 5B6 monoclonal anti-gD antibody (0.5 μ g/ml in 50 mM carbonate buffer, pH 9.6, 100 μ l/well) was decanted, tamped on a paper towel and blocked with 150 μ l/well of Block Buffer [PBS containing 0.5 % BSA (Intergen Company, Purchase, NY) and 0.01 % thimerosal] for 60 min. at room temperature with gentle agitation. After 60 minutes, the anti-gD 5B6 coated plate was washed 6 times with wash buffer (PBS containing 0.05 % Tween-20 and 0.01 % thimerosal) using an automated plate washer (ScanWasher 300, Skatron Instruments, Inc, Sterling, VA).
- The lysate containing solubilized Rse.gD from the cell-culture microtiter well was transferred (85 μ l/well) to anti-gD 5B6 coated and blocked ELISA well and was incubated for 2 h at room temperature with gentle agitation. The unbound Rse.gD was removed by washing with wash buffer and 100 μ l of biotinylated 4G10 (anti-phosphotyrosine) diluted 1:2000 in dilution buffer (PBS containing 0.5 % BSA, 0.05 % Tween-20, 5 mM EDTA, and 0.01 % thimerosal), i.e. 400 pg/ml was added to each well. After incubation for 2 h at room temperature the plate was washed and 100 μ l of HRPO-conjugated streptavidin (Zymed Laboratories, S. San Francisco, CA)

diluted 1:10000 in dilution buffer was added to each well. The plate was incubated for 30 minutes at room temperature with gentle agitation. The free avidin-conjugate was washed away and 100 μ l freshly prepared substrate solution (tetramethyl benzidine [TMB]; 2-component substrate kit; Kirkegaard and Perry, Gaithersburg, MD) was added to each well. The reaction was allowed to proceed for 10 minutes, after which the color development was stopped by the addition of 100 μ l/well 1.0 M H_3PO_4 . The absorbance at 450 nm was read with a reference wavelength of 650 nm ($ABS_{450/650}$), using a vmax plate reader (Molecular Devices, Palo Alto, CA) controlled with a Macintosh Centris 650 (Apple Computers, Cupertino, CA) and DeltaSoft software (BioMetallics, Inc, Princeton, NJ).

The standard curve shown in Figure 10 was generated by stimulating Rse.gD transformed CHO cells with 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200 or 0 diluted, anti-Rse agonist antibody (19B) and presented as 1/dilution anti-Rse agonist antibody (19B) vs. mean $ABS_{450/650} \pm sd$ using the DeltaSoft program.

The results presented in this example demonstrate that the KIRA ELISA is a useful method for assaying ligand activation of a receptor construct having a carboxyl terminal flag polypeptide, e.g., activation of Rse.gD. Levels of receptor activation in terms of tyrosine phosphorylation are easily quantified and an EC_{50} for a given ligand (e.g. an agonist antibody for the receptor) is readily determined.

EXAMPLE 3

KIRA ELISA of the trk A, B and C Receptors

Neurotrophins belong to a family of small, basic proteins which play a crucial role in the development and maintenance of the nervous system. The first identified and probably best understood member of this family is nerve growth factor (NGF). See U.S. Patent No. 5,169,762, issued December 8, 1992. Recently, sequentially related but distinct polypeptides with similar functions to NGF have been identified. For example, brain-derived neurotrophic factor (BDNF), now also referred to as neurotrophin-2 (NT2), was cloned and sequenced by Leibrock et al. (*Nature*, 341: 149-152 [1989]). Several groups identified a neurotrophic factor originally called neuronal factor (NF), and now referred to as neurotrophin-3 (NT3). (Ernfors et al., *Proc. Natl. Acad. Sci. USA*, 87: 5454-5458 [1990]; Höhn et al., *Nature*, 344: 339 [1990]; Maisonpierre et al., *Science*, 247: 1446 [1990]; Rosenthal et al., *Neuron*, 4: 767 [1990]; Jones and Reichardt, *Proc. Natl. Acad. Sci.*

USA, 87: 8060-8064 [1990]; Kaisho et al., FEBS Lett., 266: 187 [1990]).
Neurotrophins-4 and -5 (NT4 and NT5) have been recently added to the family
(Hallbook et al., Neuron, 6: 845-858 [1991]; Berkmeier et al., Neuron, 7:
857-866 [1991]; Ip et al., Proc. Natl. Acad. Sci. USA, 89: 3060-3064
5 [1992]).

Neurotrophins, similarly to other polypeptide growth factors, affect
their target cells through interactions with cell surface rPTKs (called Trk
receptors). The first member of the trk receptor family, trkA, was
initially identified as the result of an oncogenic transformation caused
10 by the translocation of tropomyosin sequences onto its catalytic domain.
Later work identified trkA as a signal transducing receptor for NGF.
Subsequently, two other related receptors, mouse and rat trkB (Klein et
al., EMBO J., 8: 3701-3709 [1989]; Middlemas et al., Mol. Cell. Biol., 11:
143-153 [1991]; EP 455,460 published 6 November 1991) and porcine, mouse
15 and rat trkC (Lamballe et al., Cell, 66: 967-979 [1991]; EP 522,530
published 13 January 1993), were identified as members of the trk receptor
family. The structures of the trk receptors are quite similar, but
alternate splicing increases the complexity of the family by giving rise
to two known forms of trkA, three known forms of trkB (two without
20 functional tyrosine kinase domains) and at least four forms of trkC
(several without functional tyrosine kinase domain, and two with small
inserts in the tyrosine kinase domain). Human trk A, B and C receptor
sequences are disclosed in U.S. Patent application Serial No. 08/215,139,
filed March 18, 1994, specifically incorporated herein by reference.

25 The following KIRA ELISA was performed using trk A, B and C receptor
constructs having amino-terminal flag polypeptides.

(i) Capture agent preparation

Monoclonal anti-gD (clone 5B6) was produced against a peptide from
Herpes simplex virus glycoprotein D as discussed above in Example 2. The
30 purified stock preparation was adjusted to 3.0mg/ml in phosphate buffered
saline (PBS), pH 7.4 and 1.0ml aliquots were stored at -20°C.

(ii) Anti-phosphotyrosine antibody preparation

Monoclonal anti-phosphotyrosine, clone 4G10, was purchased from
Upstate Biologicals, Inc (UBI, Lake Placid, NY) and biotinylated using
35 long-arm biotin-N-hydroxysuccinamide (Biotin-X-NHS, Research Organics,
Cleveland, OH).

(iii) Ligands

Nerve growth factor (NGF), neurotrophin 3 (NT3), and neurotrophin 5 (NT5) were prepared by recombinant techniques using the sequence data provided for each of these proteins in the above-mentioned references. The purified NGF, NT3 and NT5 were stored at 4°C as stock solutions (180µM, 8.8µM and 26.9µM, respectively) in PBS, pH 7.5.

(iv) Preparation of gD.trk nucleic acid

In order to express the various trk receptors with gD flags (i.e. gD.trk constructs), DNA constructs were made which encoded the signal and epitope of gD (see Paborsky et al., *supra*) fused to the amino terminus of the various trk receptors. These were made by inserting the trk receptor and gD sequences into pRK5 or pRK7 (Suva et al., *Science*, 237: 893-896 [1987]) using standard molecular biology techniques, to generate the constructs shown in Figures 12-14. In addition to the gD.trk constructs, constructs were also made to express gD tagged trk.IgG fusion proteins (i.e., gD.trk.IgG). DNA constructs encoding the chimeras of trk extracellular domain and IgG-1 Fc domains were made with the Fc region clones of human IgG-1 (Ashkenazi et al., *Immunoadhesins Intern. Rev. Immunol.*, 10: 219-227 [1993]). More specifically, the source of the IgG-1 encoding sequence was the CD4-IgG-1 expression plasmid pRKCD4₂Fc₁ (Capon et al., *Nature*, 334: 525 [1989]; Byrn et al., *Nature*, 344: 667 [1990]) containing a cDNA sequence encoding a hybrid polypeptide consisting of residues 1-180 of the mature human CD4 protein fused to human IgG-1 sequences beginning at aspartic acid 216 (taking amino acid 114 as the first residue of the heavy chain constant region; Kabat et al., *Sequences of Proteins of Immunological Interest* 4th ed. [1987]), which is the first residue of the IgG-1 hinge after the cysteine residue involved in heavy-light chain bonding, and ending with residues 441 to include the CH2 and CH3 Fc domains of IgG-1. The CD4-encoding sequence was deleted from the expression plasmid pRKCD4₂Fc₁ and the vector was fused to DNA encoding the trk receptors, with the splice between aspartate 216 of the IgG-1 and valine 402 of trkA, threonine 422 of trkB, or threonine 413 of trkC. The gD tag was added to the amino terminus of each trk.IgG in the same way as for the gD.trk constructs.

(v) Cell transformation

Human embryonic kidney 293 cells (obtained from ATCC, Rockville, MD) were transiently transfected with the nucleic acid encoding gD.trk.IgG using a calcium phosphate protocol (Gorman, *DNA Cloning: A Practical Approach* [Glover, D., ed.] Vol II: 143-190, IRL Press, Washington DC).

After twelve hours, the transformed cells were rinsed three times with serum free F12/DMEM 50:50 media (Gibco) and then serum free media was added for a 48 hour collection.

Cell lines stably expressing each of the gD.trk constructs were made by co-transfecting dp12.CHO cells (EP 307,247 published 15 March 1989) with the pRK plasmids encoding the gD tagged trk receptors and a plasmid encoding DHFR, again using calcium phosphate mediated transfection.

The media mentioned above (having the gD.trk.IgG) was used without further purification in binding assays to assess the effects of the presence of the gD flag polypeptide on neurotrophin binding to the gD.trk.IgG polypeptides. DNA encoding untagged trk.IgG polypeptide was run in parallel as a control. trk.IgG and gD tagged trk.IgG containing cell supernatants were prepared as described and used in competitive displacement assays with the appropriate iodinated neurotrophin. NGF is used as ligand for trkA, NT5 is used as ligand for trkB, and NT3 is used as a ligand for trkC. A summary of the results obtained is shown in the following table.

TABLE 1

Binding of Neurotrophins to trk.IgG

		IC50 without gD	IC50 with gD
20	trkA	68.4+/-11.9 pM	68.8+/-3.0 pM
	trkB	31.1+/-15.6 pM	12.1+/-18 pM
	trkC	31.1+/-1.1 pM	30.2+/-0.7 pM

(vi) Selection of transformed cells for use in the KIRA ELISA

It was apparent from the preceding experiment that there was no observable change in the affinity of interaction of neurotrophins with their receptor due to the presence of the gD flag polypeptide on the amino terminus. Based on this result, cells were transformed with the gD.trk constructs for use in the KIRA ELISA using the techniques described in the previous section.

After two days, dp12.CHO cells (EP 307,247 published 15 March 1989) transformed with gD.trk constructs were selected for by growth in media without GHT, and after two weeks, growing cells were sorted by FACS analysis using the 5B6 monoclonal to select cells expressing the gD flag polypeptide on their surface. gD positive cells were cloned by plating at limiting dilution and resultant colonies were then rescreened by FACS

analysis (using the anti-gD 5B6 monoclonal antibody), neurotrophin binding (as discussed above), tyrosine phosphorylation indicated by Western blot using an anti-phosphotyrosine antibody, gD expression by Western blot using the anti-gD 5B6 antibody, and immunocytochemistry using the 5B6 antibody.

- 5 Clones which were positive were then recloned by limiting dilution and were subjected to the KIRA ELISA as described below.

(vii) Media

- Cells were grown in F12/DMEM 50:50 (Gibco/BRL, Life Technologies, Grand Island, NY). The media was supplemented with 10% diafiltered FBS
10 (HyClone, Logan, Utah), 25mM HEPES and 2mM L-glutamine.

(viii) KIRA ELISA

- gD.trk transformed dp12.CHO cells (EP 307,247 published 15 March 1989) were seeded (5×10^4 per well) in a flat-bottom-96 well culture plate in 100 μ l media and cultured overnight at 37°C in 5% CO₂. The following
15 morning the well supernatants were decanted, and the plates were lightly tamped on a paper towel. 100 μ l of media containing either experimental samples or the recombinant purified NGF, NT3, or NT5 standards (3000, 1000, 333, 111, 37, 12, 4, and 0 pM) was then added to each well. The cells were stimulated at 37°C for 30 min., the well supernatants were decanted, and
20 the plates were once again lightly tamped on a paper towel. To lyse the cells and solubilize the receptors, 100 μ l of lysis buffer was added to each well. Lysis buffer consisted of 150 mM NaCl containing 50 mM HEPES (Gibco), 0.5 % Triton-X 100 (Gibco), 0.01 % thimerosal, 30 KIU/ml aprotinin (ICN Biochemicals, Aurora, OH), 1mM 4-(2-aminoethyl)-benzenesulfonyl
25 fluoride hydrochloride (AEBSF; ICN Biochemicals), 50 μ M leupeptin (ICN Biochemicals), and 2 mM sodium orthovanadate (Na₃VO₄; Sigma Chemical Co, St. Louis, MO). pH 7.5. The plate was then agitated gently on a plate shaker (Bellco Instruments, Vineland, NJ) for 60 min. at room temperature.

- While the cells were being solubilized, an ELISA microtiter plate
30 (Nunc Maxisorp, Inter Med, Denmark) coated overnight at 4°C with the 5B6 monoclonal anti-gD antibody (0.5 μ g/ml in 50 mM carbonate buffer, pH 9.6, 100 μ l/well) was decanted, tamped on a paper towel and blocked with 150 μ l/well of Block Buffer [PBS containing 0.5 % BSA (Intergen Company, Purchase, NY) and 0.01 % thimerosal] for 60 min. at room temperature with
35 gentle agitation. After 60 minutes, the anti-gD 5B6 coated plate was washed 6 times with wash buffer (PBS containing 0.05 % Tween-20 and 0.01 % thimerosal) using an automated plate washer (ScanWasher 300, Skatron Instruments, Inc, Sterling, VA).

The lysate containing solubilized gD.trk from the cell-culture microtiter well was transferred (85 μ l/well) to anti-gD 5B6 coated and blocked ELISA well and was incubated for 2 h at room temperature with gentle agitation. The unbound gD.trk was removed by washing with wash buffer and 100 μ l of biotinylated 4G10 (anti-phosphotyrosine) diluted 1:2000 in dilution buffer (PBS containing 0.5 % BSA, 0.05 % Tween-20, 5 mM EDTA, and 0.01 % thimerosal), i.e., 400pg/ml, was added to each well. After incubation for 2 h at room temperature the plate was washed and 100 μ l of HRP-conjugated streptavidin (Zymed Laboratories, S. San Francisco, CA) diluted 1:10000 in dilution buffer was added to each well. The plate was incubated for 30 minutes at room temperature with gentle agitation. The free avidin-conjugate was washed away and 100 μ l freshly prepared substrate solution (tetramethyl benzidine; 2-component substrate kit; Kirkegaard and Perry, Gaithersburg, MD) was added to each well. The reaction was allowed to proceed for 10 minutes, after which the color development was stopped by the addition of 100 μ l/well 1.0 M H_3PO_4 . The absorbance at 450 nm was read with a reference wavelength of 650 nm ($ABS_{450/650}$), using a vmax plate reader (Molecular Devices, Palo Alto, CA) controlled with a Macintosh Centris 650 (Apple Computers, Cupertino, CA) and DeltaSoft software (BioMetallics, Inc, Princeton, NJ).

The standard curves shown in Figures 15A-15C were generated by stimulating gD.trk transformed CHO cells with 3000, 1000, 333, 111, 37, 12, 4, and 0 pM NGF, NT3 or NT5 and were presented as pM neurotrophin vs. mean $ABS_{450/650} \pm sd$ using the DeltaSoft program. Sample concentrations were obtained by interpolation of their absorbance on the standard curve and are expressed in terms of pM neurotrophin activity.

The results presented in this example demonstrate that the KIRA ELISA is a useful method for assaying ligand activation of a receptor construct having an amino terminal flag polypeptide, e.g., activation of gD.trk receptor constructs. Levels of receptor activation in terms of tyrosine phosphorylation are easily quantified and an EC_{50} for a given ligand is readily determined.

EXAMPLE 4

KIRA ELISA of the MPL/Rse Chimeric Receptor

The human MPL receptor has been disclosed by Vigon et al., PNAS, USA 89:5640-5644 (1992). A chimeric receptor comprising the ECD of the MPL receptor and the TM and ICD of Rse (Mark et al., *supra*) with a carboxyl-

terminal flag polypeptide (i.e. Rse.gD; see Example 2) was subjected to the KIRA ELISA described herein. The experimental procedure is outlined below. See also Figs. 16 and 17.

(i) Capture agent preparation

- 5 Monoclonal anti-gD (clone 5B6) was produced against a peptide from Herpes simplex virus glycoprotein D (Paborsky et al., Protein Engineering 3(6):547-553 [1990]). The purified stock preparation was adjusted to 3.0mg/ml in phosphate buffered saline (PBS), pH 7.4 and 1.0ml aliquots were stored at -20°C.

10 (ii) Anti-phosphotyrosine antibody preparation

Monoclonal anti-phosphotyrosine, clone 4G10, was purchased from UBI (Lake Placid, NY) and biotinylated using long-arm biotin-N-hydroxysuccinamide (Biotin-X-NHS, Research Organics, Cleveland, OH).

(iii) Ligand

- 15 The MPL ligand [de Sauvage et al., Nature 369: 533-538 (1994)] was prepared by recombinant techniques. The purified MPL ligand was stored at 4°C as a stock solution.

(iv) Preparation of MPL/Rse.gD nucleic acid

- The expression plasmid pSV.ID.Rse.gD produced as described in Example 20 2 above was modified to produce plasmid pSV.ID.M.tmRd6 which contained the coding sequences of the ECD of human MPL (amino acids 1-491) fused to the transmembrane domain and intracellular domain of Rse.gD (amino acids 429-911). Synthetic oligonucleotides were used to join the coding sequence of a portion of the extracellular domain of human MPL to a portion of the Rse coding sequence in a two step PCR cloning reaction as described by Mark et 25 al. in J. Biol. Chem. 267: 26166-26171 (1992). Primers used for the first PCR reaction were M1 (5'-TCTCGCTACCGTTTACAG - SEQ ID NO:12) and M2 (5'-CAGGTACCCACCAGGCGGTCTCGGT - SEQ ID NO: 13) with a MPL cDNA template and R1 (5'-GGGCCATGACACTGTCAA - SEQ ID NO: 14) and R2 (5'-GACCGCCACCGAGACCGCCTGGTGGGTACCTGTGGTCTT - SEQ ID NO: 15) with a Rse cDNA 30 template. The PvuII-SmaI portion of this fusion junction was used for the construction of the full-length chimeric receptor.

(v) Cell transformation

- dp12.CHO cells (EP 307,247 published 15 March 1989) were 35 electroporated with pSV.ID.M.tmRd6 which had been linearized at a unique NotI site in the plasmid backbone. The DNA was ethanol precipitated after phenol/chloroform extraction and was resuspended in 20µl 1/10 Tris EDTA. Then, 10µg of DNA was incubated with 10⁷ CHO.dp12 cells in 1 ml of PBS on

ice for 10 min. before electroporation at 400 volts and 330 μ f. Cells were returned to ice for 10 min. before being plated into non-selective medium. After 24 hours cells were fed nucleoside-free medium to select for stable DHFR⁺ clones.

5 (vi) Selection of transformed cells for use in the KIRA ELISA

Clones expressing MPL/Rse.gD were identified by western-blotting of whole cell lysates post-fractionation by SDS-PAGE using the antibody 5B6 which detects the gD epitope tag.

(vii) Media

10 Cells were grown in F12/DMEM 50:50 (Gibco/BRL, Life Technologies, Grand Island, NY). The media was supplemented with 10% diafiltered FBS (HyClone, Logan, Utah), 25mM HEPES and 2mM L-glutamine.

(viii) KIRA ELISA

MPL/Rse.gD transformed dp12.CHO cells were seeded (3x10⁴ per well) in
15 the wells of a flat-bottom-96 well culture plate in 100 μ l media and cultured overnight at 37°C in 5% CO₂. The following morning the well supernatants were decanted, and the plates were lightly tamped on a paper towel. 50 μ l of media containing either experimental samples or 200, 50, 12.5, 3.12, 0.78, 0.19, 0.048 or 0 ng/ml MPL ligand was then added to each
20 well. The cells were stimulated at 37°C for 30 min., the well supernatants were decanted, and the plates were once again lightly tamped on a paper towel. To lyse the cells and solubilize the chimeric receptors, 100 μ l of lysis buffer was added to each well. Lysis buffer consisted of 150 mM NaCl containing 50 mM HEPES (Gibco), 0.5 % Triton-X 100 (Gibco), 0.01 %
25 thimerosal, 30 KIU/ml aprotinin (ICN Biochemicals, Aurora, OH), 1mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF; ICN Biochemicals), 50 μ M leupeptin (ICN Biochemicals), and 2 mM sodium orthovanadate (Na₃VO₄; Sigma Chemical Co, St. Louis, MO), pH 7.5. The plate was then agitated gently on a plate shaker (Bellco Instruments, Vineland,
30 NJ) for 60 min. at room temperature.

While the cells were being solubilized, an ELISA microtiter plate (Nunc Maxisorp, Inter Med, Denmark) coated overnight at 4°C with the 5B6 monoclonal anti-gD antibody (5.0 μ g/ml in 50 mM carbonate buffer, pH 9.6, 100 μ l/well) was decanted, tamped on a paper towel and blocked with 150
35 μ l/well of Block Buffer [PBS containing 0.5 % BSA (Intergen Company, Purchase, NY) and 0.01 % thimerosal] for 60 min. at room temperature with gentle agitation. After 60 minutes, the anti-gD 5B6 coated plate was washed 6 times with wash buffer (PBS containing 0.05 % Tween-20 and 0.01 %

thimerosal) using an automated plate washer (ScanWasher 300, Skatron Instruments, Inc, Sterling, VA).

The lysate containing solubilized MPL/Rse.gD from the cell-culture microtiter well was transferred (85 μ l/well) to anti-gD 5B6 coated and
5 blocked ELISA well and was incubated for 2 h at room temperature with gentle agitation. The unbound MPL/Rse.gD was removed by washing with wash buffer and 100 μ l of biotinylated 4G10 (anti-phosphotyrosine) diluted 1:18000 in dilution buffer (PBS containing 0.5 % BSA, 0.05 % Tween-20, 5 mM EDTA, and 0.01 % thimerosal), i.e. 56 ng/ml was added to each well.
10 After incubation for 2 h at room temperature the plate was washed and 100 μ l of HRP-conjugated streptavidin (Zymed Laboratories, S. San Francisco, CA) diluted 1:60000 in dilution buffer was added to each well. The plate was incubated for 30 minutes at room temperature with gentle agitation. The free avidin-conjugate was washed away and 100 μ l freshly prepared
15 substrate solution (tetramethyl benzidine [TMB]; 2-component substrate kit; Kirkegaard and Perry, Gaithersburg, MD) was added to each well. The reaction was allowed to proceed for 10 minutes, after which the color development was stopped by the addition of 100 μ l/well 1.0 M H_3PO_4 . The absorbance at 450 nm was read with a reference wavelength of 650 nm
20 ($ABS_{450/650}$), using a vmax plate reader (Molecular Devices, Palo Alto, CA) controlled with a Macintosh Centris 650 (Apple Computers, Cupertino, CA) and DeltaSoft software (BioMetallics, Inc, Princeton, NJ).

The results demonstrated that MPL ligand was able to activate the MPL/Rse.gD chimeric receptor in a concentration-dependent and ligand-
25 specific manner.

CLAIMS

1. A method for measuring autophosphorylation of a tyrosine kinase receptor comprising the steps of:
 - (a) coating a first solid phase with a homogeneous population of eukaryotic cells so that the cells adhere to the first solid phase, wherein, positioned in their membranes, the cells have a receptor construct comprising a flag polypeptide and the tyrosine kinase receptor;
 - (b) exposing the adhering cells to an analyte;
 - 10 (c) solubilizing the adhering cells, thereby releasing cell lysate therefrom;
 - (d) coating a second solid phase with a capture agent which binds specifically to the flag polypeptide so that the capture agent adheres to the second solid phase;
 - 15 (e) exposing the adhering capture agent to the cell lysate obtained in step (c) so that the receptor construct adheres to the second solid phase;
 - (f) washing the second solid phase so as to remove unbound cell lysate;
 - 20 (g) exposing the adhering receptor construct to an anti-phosphotyrosine antibody which identifies phosphorylated tyrosine residues in the tyrosine kinase receptor; and
 - (h) measuring binding of the anti-phosphotyrosine antibody to the adhering receptor construct.
- 25 2. The method of claim 1 wherein the cells are transformed with nucleic acid encoding the receptor construct prior to step (a).
3. The method of claim 1 wherein the cells comprise a mammalian cell line.
4. The method of claim 1 wherein the cells are adherent.
- 30 5. The method of claim 1 wherein the capture agent comprises a capture antibody.

6. The method of claim 1 wherein the first solid phase comprises a well of a first assay plate.
7. The method of claim 6 wherein the first assay plate is a microtiter plate.
- 5 8. The method of claim 6 wherein between about 1×10^4 to 3×10^5 cells are added to the well in step (a).
9. The method of claim 1 wherein the second solid phase comprises a well of a second assay plate.
- 10 10. The method of claim 1 wherein the cell lysate is not concentrated or clarified prior to step (e).
11. The method of claim 6 wherein step (c) comprises adding a lysis buffer to the well of the first assay plate and gently agitating the first assay plate.
12. The method of claim 11 wherein the lysis buffer comprises a solubilizing detergent.
- 15 13. The method of claim 1 wherein the anti-phosphotyrosine antibody is labelled.
14. The method of claim 13 wherein the label comprises an enzyme which is exposed to a color reagent and the color change of the color reagent is determined in step (h).
- 20 15. The method of claim 1 wherein the flag polypeptide is fused to the amino terminus of the tyrosine kinase receptor.
16. The method of claim 15 wherein the tyrosine kinase receptor is a trk A receptor, trk B receptor or trk C receptor.
- 25 17. The method of claim 1 wherein the flag polypeptide is fused to the carboxyl terminus of the tyrosine kinase receptor.

18. The method of claim 17 wherein the tyrosine kinase receptor is the Rse receptor.
19. The method of claim 17 wherein the receptor construct comprises the extracellular domain of a receptor of interest and the intracellular domain of the Rse receptor.
5
20. The method of claim 19 wherein the receptor of interest is the MPL receptor.
21. The method of claim 20 wherein the receptor construct further comprises the transmembrane domain of the Rse receptor and the flag polypeptide comprises the gD polypeptide.
10
22. The method of claim 1 wherein the analyte comprises an agonist for the tyrosine kinase receptor.
23. The method of claim 1 wherein the analyte comprises an antagonist for the tyrosine kinase receptor.
- 15 24. The method of claim 23 wherein the antagonist competitively inhibits binding or activation of the tyrosine kinase receptor by an agonist thereto and step (b) is followed by a step wherein the adhering cells are exposed to the agonist.
- 20 25. The method of claim 1 wherein the analyte is a composition which comprises an antagonist and an agonist for the receptor and the assay measures the ability of the antagonist to bind to the agonist and thereby reduce activation of the tyrosine kinase receptor by the agonist.
26. The method of claim 1 wherein a block buffer is added to the second solid phase following step (d).
25
27. A method for measuring autophosphorylation of a tyrosine kinase receptor comprising the steps of:
 - (a) coating a well of a first assay plate with a homogeneous population of adherent cells so that the cells adhere to the

- well, wherein the cells have a tyrosine kinase receptor positioned in the cell membranes thereof;
- (b) exposing the adhering cells to an analyte;
- (c) solubilizing the adhering cells thereby releasing cell lysate therefrom;
- (d) coating a well of a second assay plate with a capture agent which binds specifically to the tyrosine kinase receptor so that the capture agent adheres to the well;
- (e) exposing the cell lysate obtained in step (c) to the adhering capture agent so that the tyrosine kinase receptor adheres to the well;
- (f) washing the well so as to remove unbound cell lysate;
- (g) exposing the adhering tyrosine kinase receptor to an anti-phosphotyrosine antibody which binds selectively to phosphorylated tyrosine residues in the tyrosine kinase receptor;
- (h) measuring binding of the anti-phosphotyrosine antibody to the adhering tyrosine kinase receptor.
28. The method of claim 27 wherein the tyrosine kinase receptor comprises the HER2 receptor.
29. A polypeptide comprising a flag polypeptide fused to the carboxyl terminus of the intracellular domain of the Rse receptor.
30. The polypeptide of claim 29 further comprising the transmembrane domain of the Rse receptor.
31. The polypeptide of claim 30 further comprising the extracellular domain of a receptor protein tyrosine kinase, other than the Rse receptor.
32. The polypeptide of claim 29 wherein the flag polypeptide comprises the gD flag.
33. A kit comprising a solid phase coated with a capture agent which binds specifically to a flag polypeptide.

34. The kit of claim 33 wherein the solid phase comprises a well of a microtiter plate.
35. The kit of claim 33 further comprising a labeled anti-phosphotyrosine antibody.
- 5 36. The kit of claim 35 wherein the label comprises an enzyme.
37. The kit of claim 33 further comprising a cell transformed with a nucleic acid encoding a receptor construct.
38. An assay for measuring phosphorylation of a kinase comprising the steps of:
- 10 (a) coating a first solid phase with a homogeneous population of eukaryotic cells so that the cells adhere to the first solid phase, wherein the cells comprise a kinase construct comprising a flag polypeptide and the kinase;
- (b) exposing the adhering cells to an analyte;
- 15 (c) solubilizing the adhering cells, thereby releasing cell lysate therefrom;
- (d) coating a second solid phase with a capture agent which binds specifically to the flag polypeptide so that the capture agent adheres to the second solid phase;
- 20 (e) exposing the adhering capture agent to the cell lysate obtained in step (c) so that the kinase construct adheres to the second solid phase;
- (f) washing the second solid phase so as to remove unbound cell lysate;
- 25 (g) exposing the adhering kinase construct to an antibody which identifies phosphorylated residues in the kinase construct; and
- (h) measuring binding of the antibody to the adhering kinase construct.
39. The assay of claim 38 wherein the kinase is a receptor.
- 30 40. The assay of claim 38 wherein the kinase is a serine-threonine kinase.

41. The assay of claim 38 which measures phosphatase activity.
42. The assay of claim 41 wherein the cells further comprise a phosphatase and the assay further comprises the step of exposing the eukaryotic cells to a phosphatase inhibitor prior to step (c).
- 5 43. The assay of claim 41 which further comprises the steps in between steps (f) and (g) of exposing the adhering kinase construct to a phosphatase and then washing the second solid phase so as to remove unbound phosphatase.

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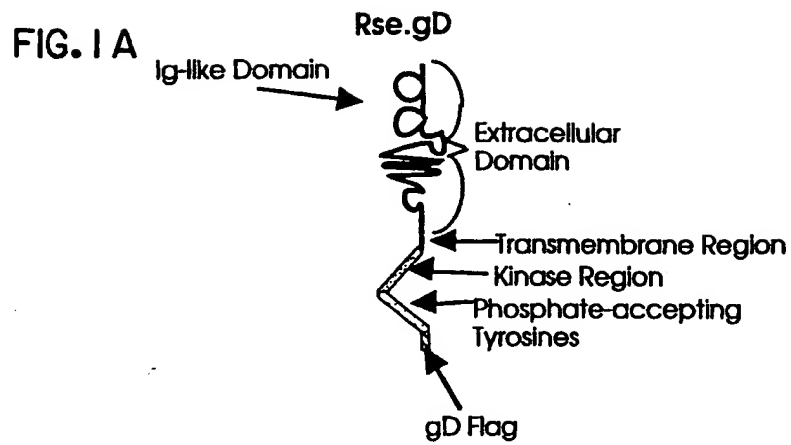
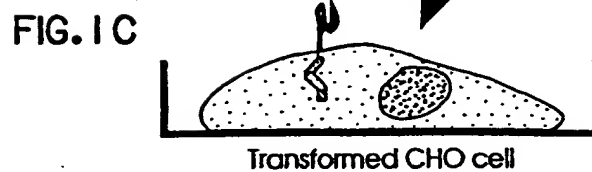
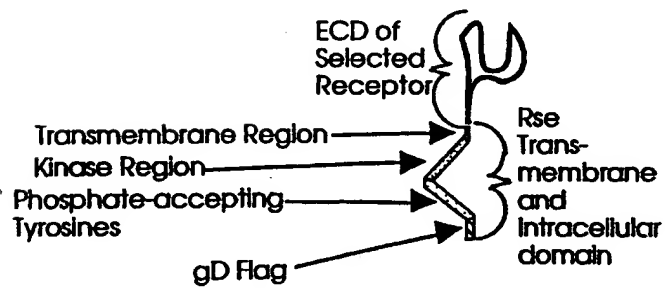


FIG. 1 B Receptor ECD/Rse.gD Chimera



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signal sequence
1 M A L R S M G R P G L P P L P L P P P P R L G L L L A A L A S L L L
1 ATGGCGCTGA GGGCGGAGCAT GGGCGGCGG GGGCTCCCGC CGTGCCGCT GCGCGCGCA CCGCGGCTG GGTGCTGCT GCGGCTCTG GCTTCTCTGC
35 * * * * * extracellular domain
L P E S A A A G L K L M G A P V K L T V S Q G Q P V K L N C S V E
101 TGTCCTCCGA GTCCGCCGC GCAGTCTGA AGCTCATGGG AGCCCGGTG AGCTGACAG TGCTTCAGGG GCAGCGGTG AAGCTCAACT GCAGTGTGA
68 G M E E P D I Q W V K D G A V V Q N L D Q L Y I P V S E Q H W I G
201 GGGATGGAG GAGCTGACA TCCAGTGGGT GAAGGATGGG GCTGTGGTCC AGAAGTTGA CCAGTTGTAC ATCCAGTCA CCGAGCAGCA CTGGATGGC
101 F L S L K S V E R S D A G R Y W C Q V E D G G E T E I S Q P V W L T
301 TTCCTCAGCC TGAAGTCAGT GGAGCGCTCT GACGCCGCC GGTACTGGTG CCAGGTGGAG GATGGGGTG AAACCGAGAT CTCCAGGCA GTGTGGCTCA
135 V E G V P F F T V E P K D L A V P P N A P F Q L S C E A V G P P E
401 CGGTAGAAGG TGTGCCATTT TTCACACTGG AGCCAAAGA TCTGGCAGTG CCACCCAATG CCCCTTTCCA ACTGTCTGT GAGGCTGTGG GTCCCCCTGA
168 P V T I V W W R G T T K I G G P A P S P S V L N V T G V T Q S T M
501 ACCTGTACC ATTGTCTGGT GGAGAGGAAC TACGAAGATC GGGGGACCG CTCCTCTCC ATCTGTTTA AATGTAACAG GGTGACCA GAGCACCATG
201 F S C E A H N L K G L A S S R T A T V H L Q A L P A A P F N I T V T
601 TTTTCTCTG AAGCTCACAA CCTAAAAGC CTGGCTCTT CTCGCACAGC CACTGTTTAC CTTCAGCAC TGCCTGCAGC CCCCTTCAAC ATCACCCTGA
235 K L S S S N A S V A W M P G A D G R A L L Q S C T V Q V T Q A P G
701 CAAAGCTTC CAGCAGCAAC GCTAGTGTGG CCTGGATGCC AGTGCTGAT GCGCGAGTC TGCTACAGTC CTGTACAGTT CAGGTGACAC AGGCCCCAGG
268 G W E V L A V V V P V P P F T C L L R D L V P A T N Y S L R V R C
801 AGCGTGGAA GTCCTGGCTG TTGTGGTCCC TGTCCTCCCGA CCTGTGCTT GCGCCTCACT ACAGCCTCAG GGTGCGCTGT
301 A N A L G P S P Y A D W V P F Q T K G L A P A S A P Q N L H A I R T
901 GCCAATGCCT TGGGGCCCTC TCCCTATGCT GACTGGGTGC CCTTTCAGAC CAAGGTCTA GCGCCAGCA CCGCTCCCCA AAACCTCCAT GCCATCCGCA
335 D S G L I L E W E V I P E A P L E G P L G P Y K L S W V Q D N G
1001 CAGATTACG CCTCATCTTG GAGTGGGAAG AAGTGATCCC CGAGGCCCTT TTGGAAGGCC CCTGGGACC CTACAAACTG TCCTGGGTTT AAGACAATGG

FIG. 2A

368 T Q D E L T V E G T R A N L T G W D P Q K D L I V R V C V S N A V
 1101 AACCCAGGAT GAGCTGACAG TGGAGGGGAC CAGGGCCCAT TTGACAGGCT GGGATCCCCA AAAGGACCTG ATCGTACGTG TGTGCGTCTC CAATGCAGTT
 transmembrane domain
 401 G C G P W S Q P L V V S S H D R A G Q Q G P P H S R T S W V P V V L
 1201 GGCTGTGGAC CCTGGAGTCA GCCACTGGTG GTCTCTTCTC ATGACCGTGC AGCCAGCAG GGCCTCTCTC ACAGCCGCAC ATCCTGGGTA CCTGTGTCTC
 intracellular domain
 435 G V L T A L V T A A A L A L I L L R K R R K E T R F G Q A F D S V
 1301 TTGGTGTGCT AACGGCCCTG GTGACGGCTG CTGCCCTGGC CTTCACTCTG CTTCGAAAGA GACGGAAAGA GACCGGTTT GGGCAAGCCT TTGACAGTGT
 468 M A R G E P A V H F R A A R S F N R E R P E R I E A T L D S L G I
 1401 CATGGCCCGG GGAGAGCCAG CCGTTCACTT CCGGGCAGCC CGTCTCTTCA ATCGAGAAAG GCCCGAGCGC ATCGAGGCCA CATTGGACAG CTTGGGCAATC
 501 S D E L K E K L E D V L I P E Q Q F T L G R M L G K G E F G S V R E
 1501 AGCGATGAAC TAAGGAAAA ACTGGAGGAT GTGCTCATCC CAGAGCAGCA GTTCAACCCTG GCCCGGATGT TGGGCAAAAG AGAGTTTGGT TCAGTGCGGG
 535 A Q L K Q E D G S F V K V A V K M L K A D I I A S S D I E E F L R
 1601 AGGCCAGCT GAAGCAAGAG GATGGCTCCT TTGTGAAGT GGCTGTGAAG ATGCTGAAG CTGACATCAT TGCCTCAAGC GACATTGAAG AGTTCCTCAG
 568 E A A C M K E F D H P H V A K L V G V S L R S R A K G R L P I P M
 1701 GGAAGCAGCT TGCATGAAGG AGTTTGACCA TCCACACGTG GCCAAACTTG TTGGGGTAAG CCTCCGGAGC AGGGCTAAAG GCCGTCTCCC CATCCCCATG
 601 V I L P F M K H G D L H A F L L A S R I G E N P F N L P L Q T L I R
 1801 GTCATCTTGC CCTTCATGAA GCATGGGGAC CTGCATGCCT TCCTGCTGCG CTCCCGGATT GGGGAGAACC CCTTTAACCT ACCCTCCAG ACCCTGATCC
 635 F M V D I A C G M E Y L S S R N F I H R D L A A R N C M L A E D M
 1901 GGTTCATGGT GGACATTGCC TCGGGCATGG AGTACCTGAG CTCTCGGAAC TTCACTCCACC GAGACCTGGC TGCTCGGAAT TGCATGCTGG CAGAGGACAT
 668 T V C V A D F G L S R K I Y S G D Y Y R Q G C A S K L P V K W L A
 2001 GACAGTGTGT GTGGCTGACT TCGGACTCTC CCGGAAGATC TACAGTGGG ACTACTATCG TCAAGGCTGT GCCTCCAAAC TGCCTGTCAA GTGGCTGGCC

FIG.2B 3/70

701 L E S L A D N L Y T V Q S D V W A F G V T M W E I M T R G Q T P Y A
 2101 CTGGAGAGCC TGSCGACAA CCTGTATACT GTGCAGAGTG ACGTGTGGG ACCATGTGG AGATCATGAC ACGTGGGCAG ACGCCATATG
 735 G I E N A E I Y N Y L I G G N R L K Q P P E C M E D V Y D L M Y Q
 2201 CTGGCATCGA AACGCTGAG ATTTACAACCT ACCTCATTTG CGGGAACCGC CTGAACACAGC CTCGGGAGTG TATGGAGGAC GTGTATGATC TCATGTACCA
 768 C W S A D P K Q R P S F T C L R M E L E N I L G Q L S V L S A S Q
 2301 GTGCTGGAGT GCTGACCCCA AGCAGCGCCC GAGCTTTACT TGTCTGCGAA TGGAACTGGA GAACATCTTG GCCCAGCTGT CTGTGCTATC TGCCAGCCAG
 801 D P L Y I N I E R A E E P T A G G S L E L P G R D Q P Y S G A G D G
 2401 GACCCCTTAT ACATCAACAT CGAGAGAGCT GAGGAGCCCA CTGCGGGAGG CAGCCTGGAG CTACCTGGCA GGGATCAGCC CTACAGTGGG GCTGGGCGATG
 835 S G M G A V G G T P S D C R Y I L T P G G L A E Q P G Q A E H Q P
 2501 GCAGTGGCAT GGAGGCGAGTG GGTGGCACTC CCAGTGA CTGCGTACATA CTCACCCCG GAGGGCTGGC TGAGCAGCCA GGCAGGCCAG AGCACCAGCC
 868 E S P L N E T Q R L L L L Q Q G L L P H S S C A D A S L K M A D P
 2601 AGAGAGTCCC CTCATATGAGA CACAGAGGCT TTTGCTGCTG CAGCAAGGSC TACTGCCACA CTCGAGCTGC GCAGATGCTA GCCTCAAGAT GGCTGATCCA
 901 N R F R G K D L P V L O
 2701 AATCGATTCC GCGGCAAGA TCTTCCGGTC CTGTAGAAGC TT

FIG.2C
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gd flag polypeptide

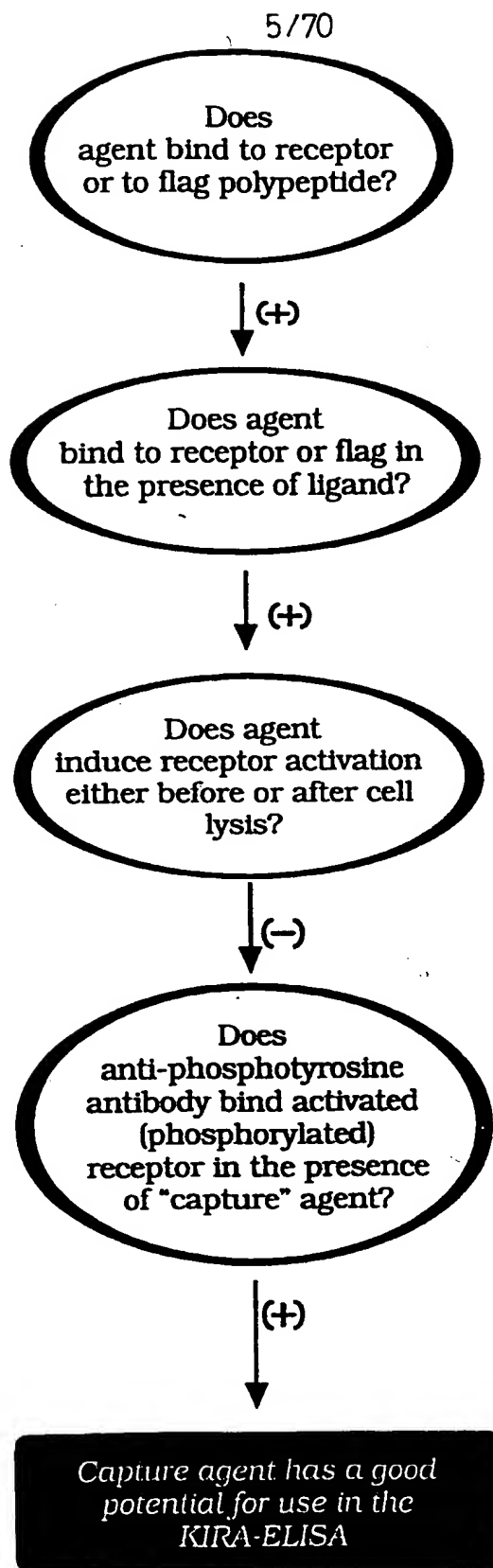


FIG.3

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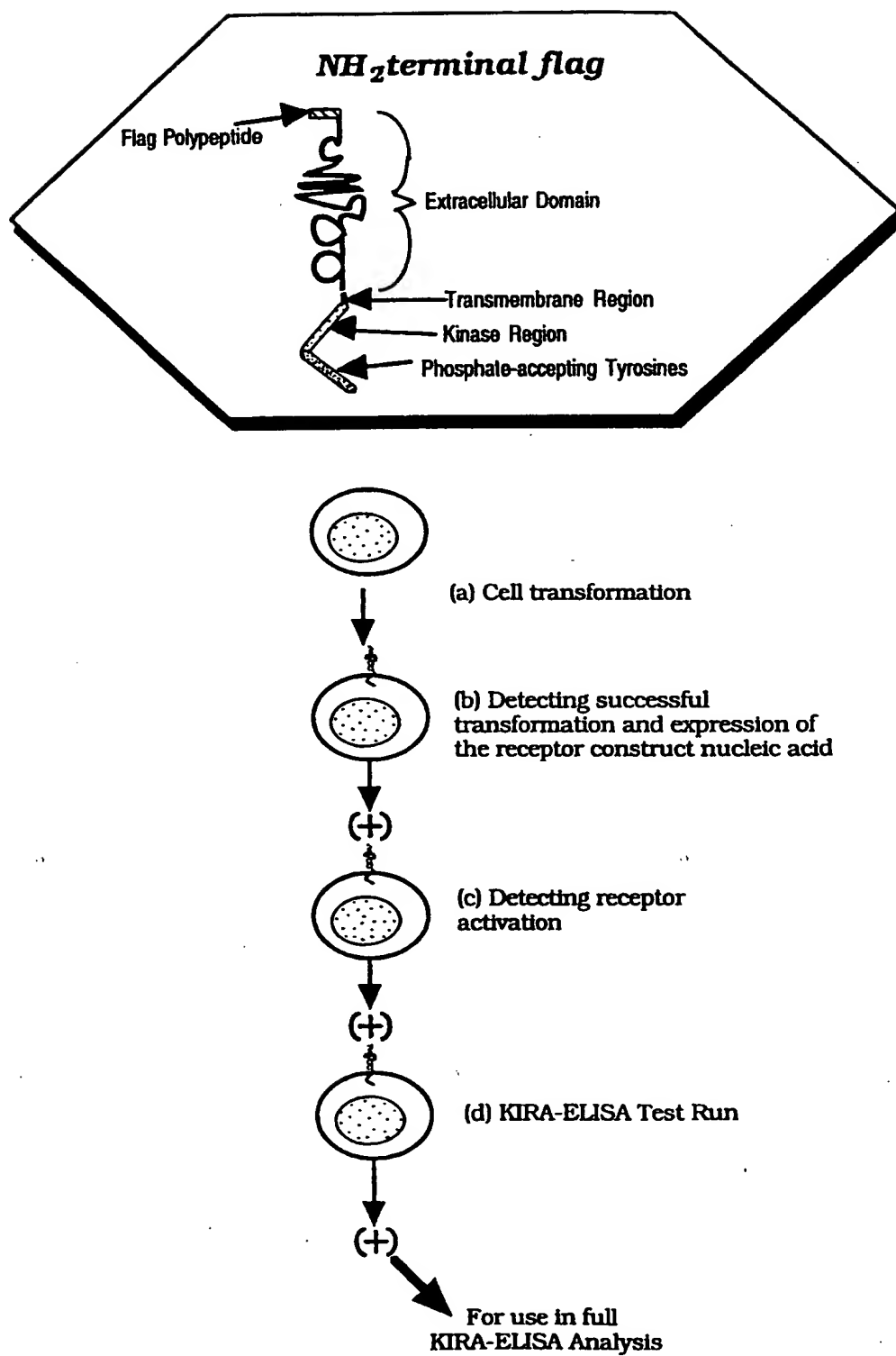


FIG.4

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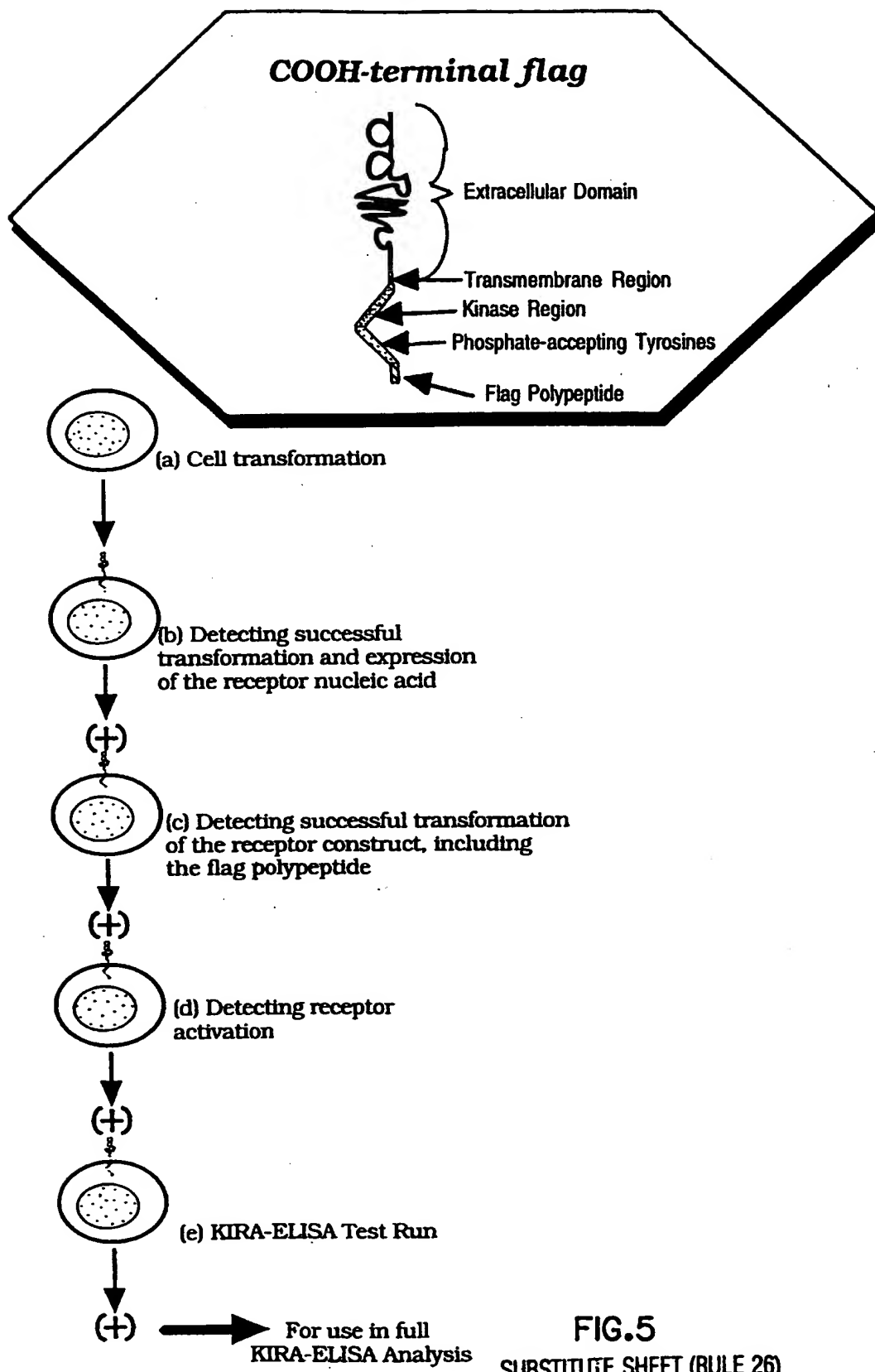
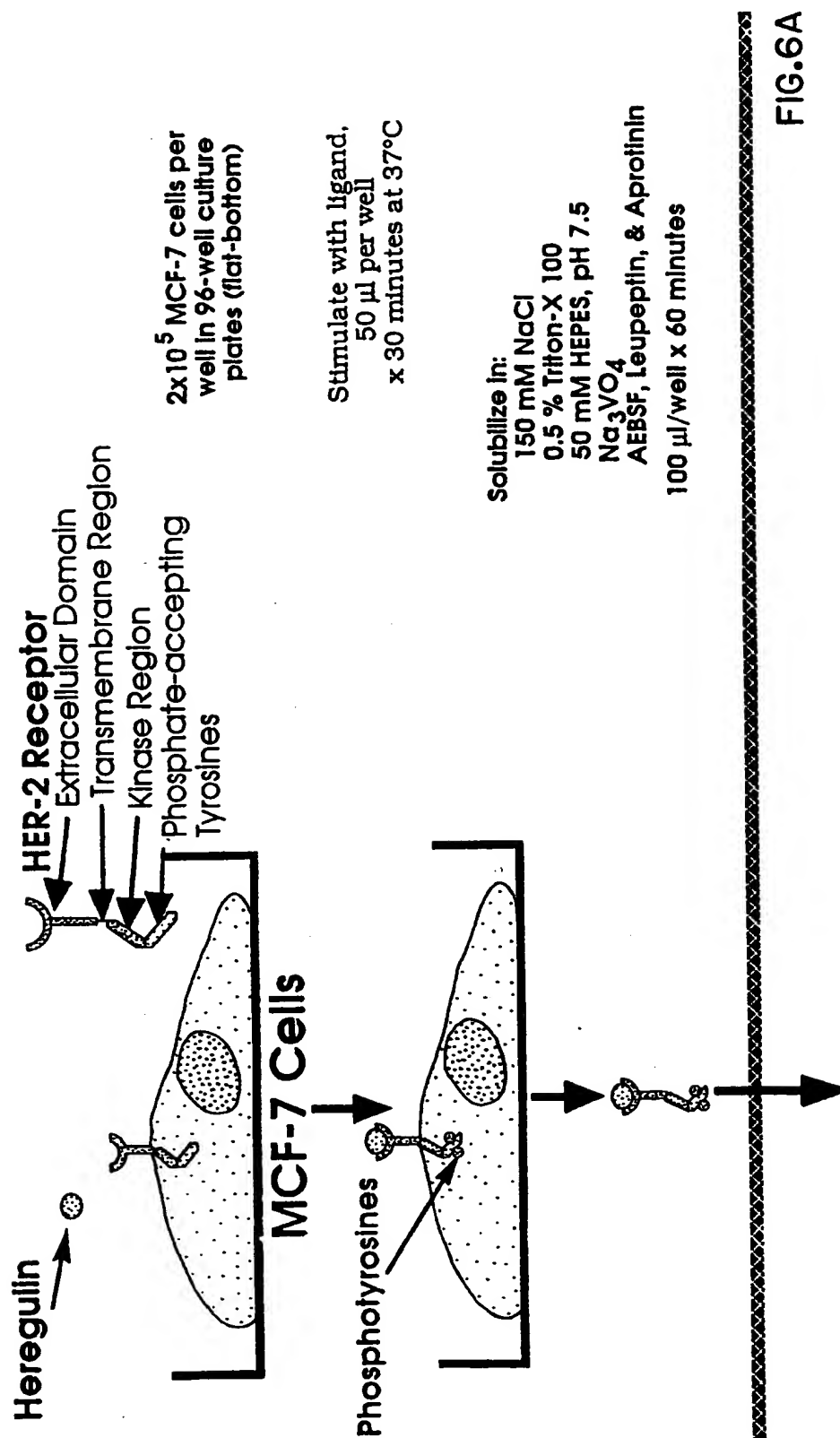


FIG.5
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ELISA 96-well plate

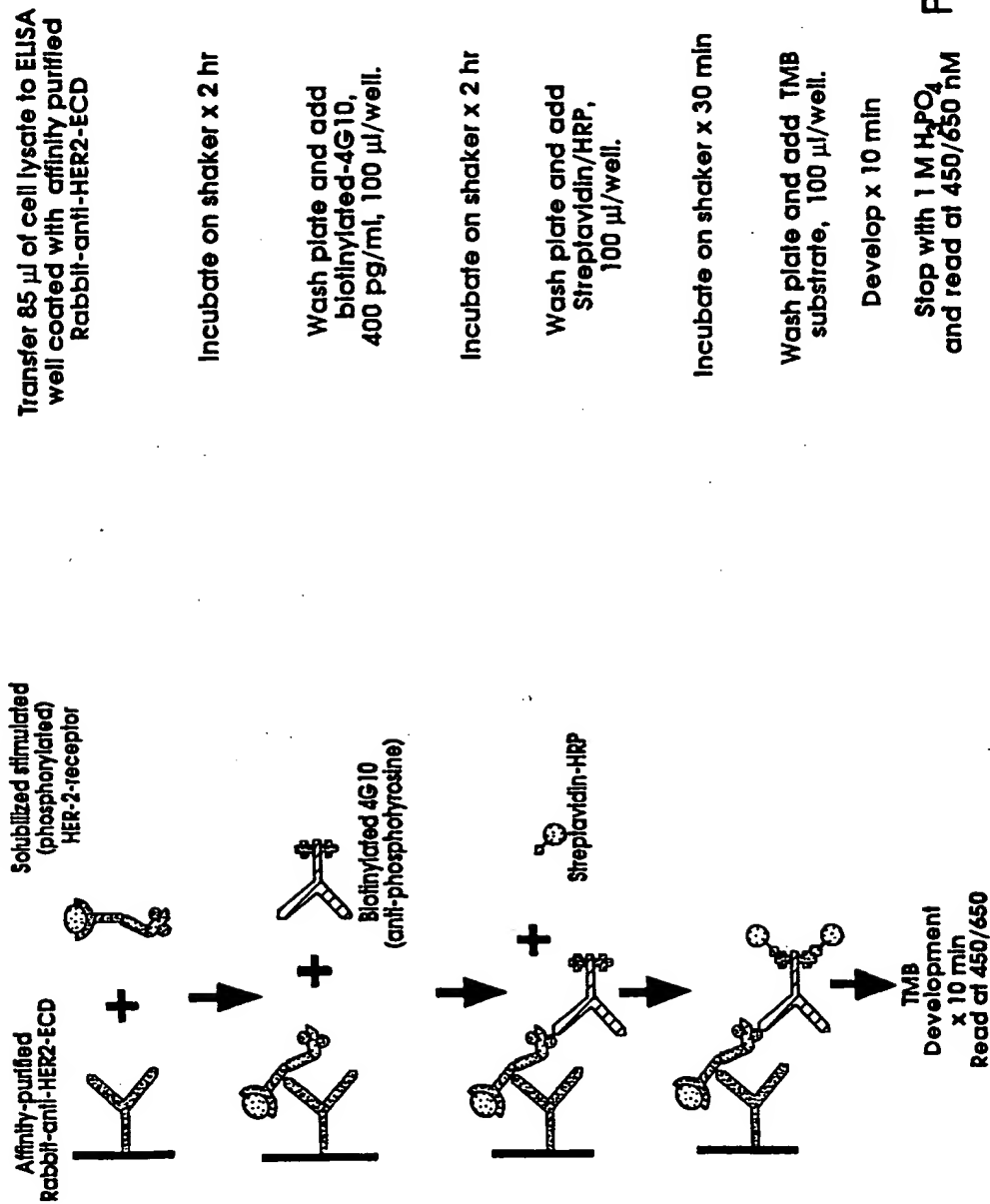
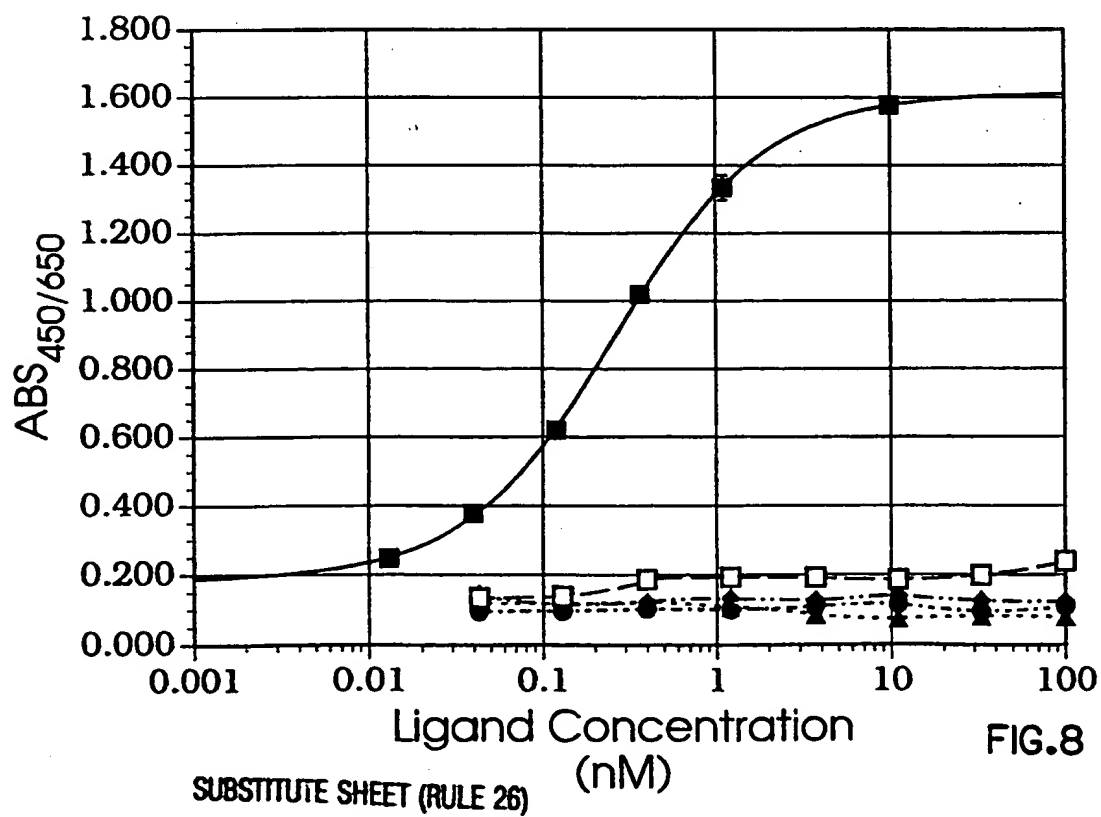
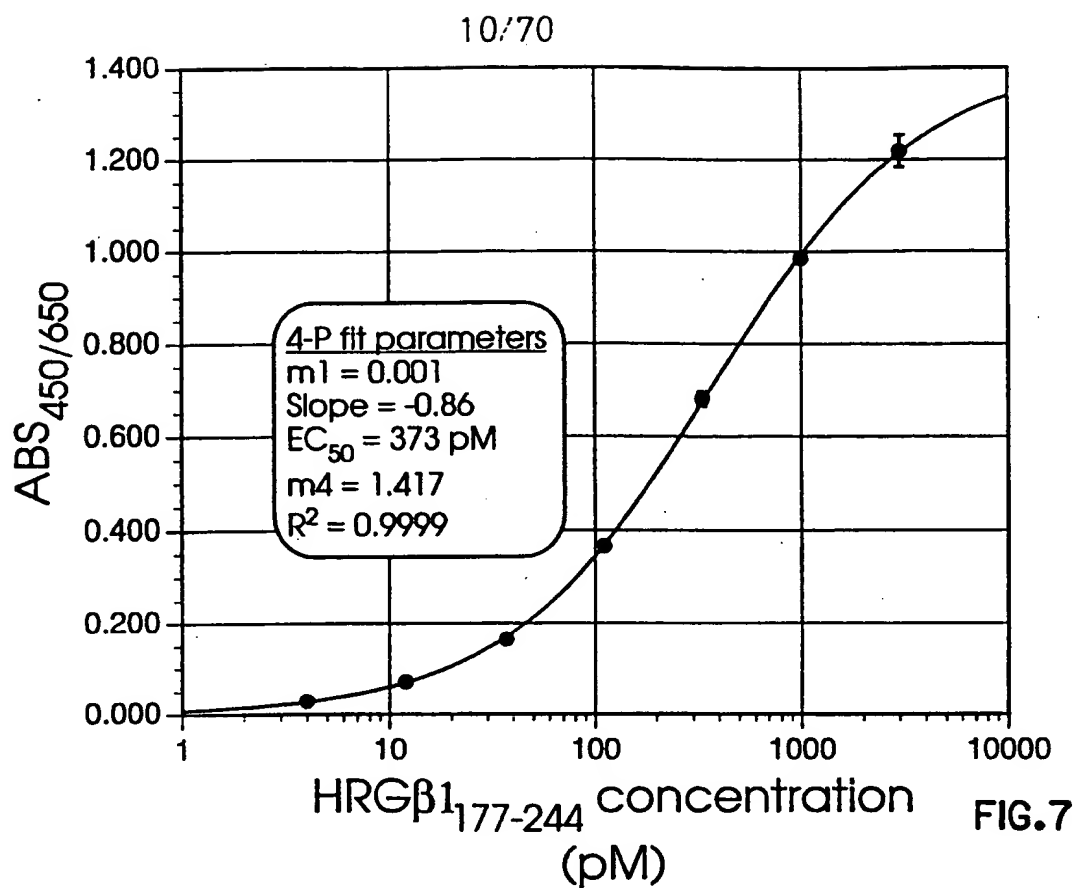
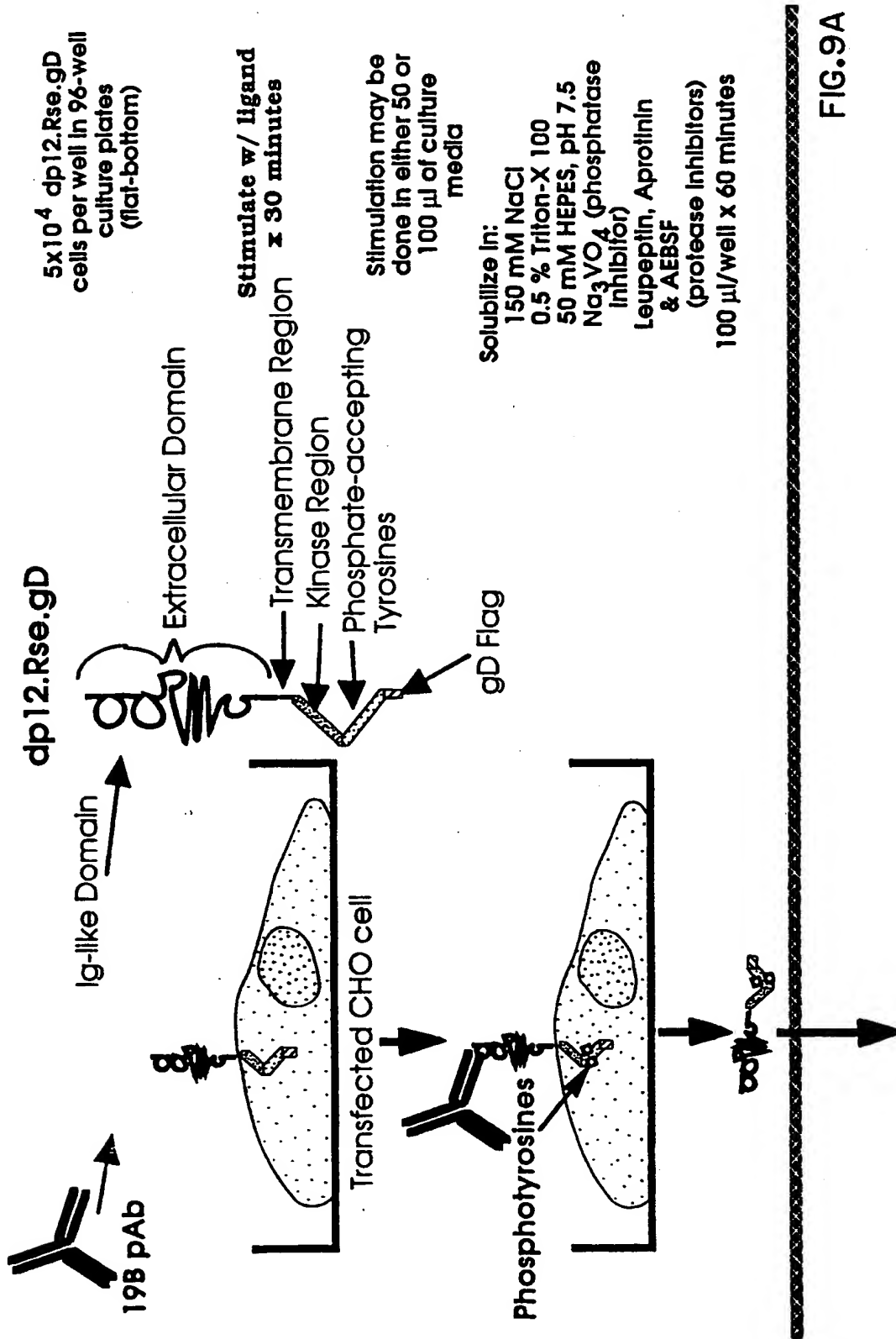


FIG.6B



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ELISA 96-well plate

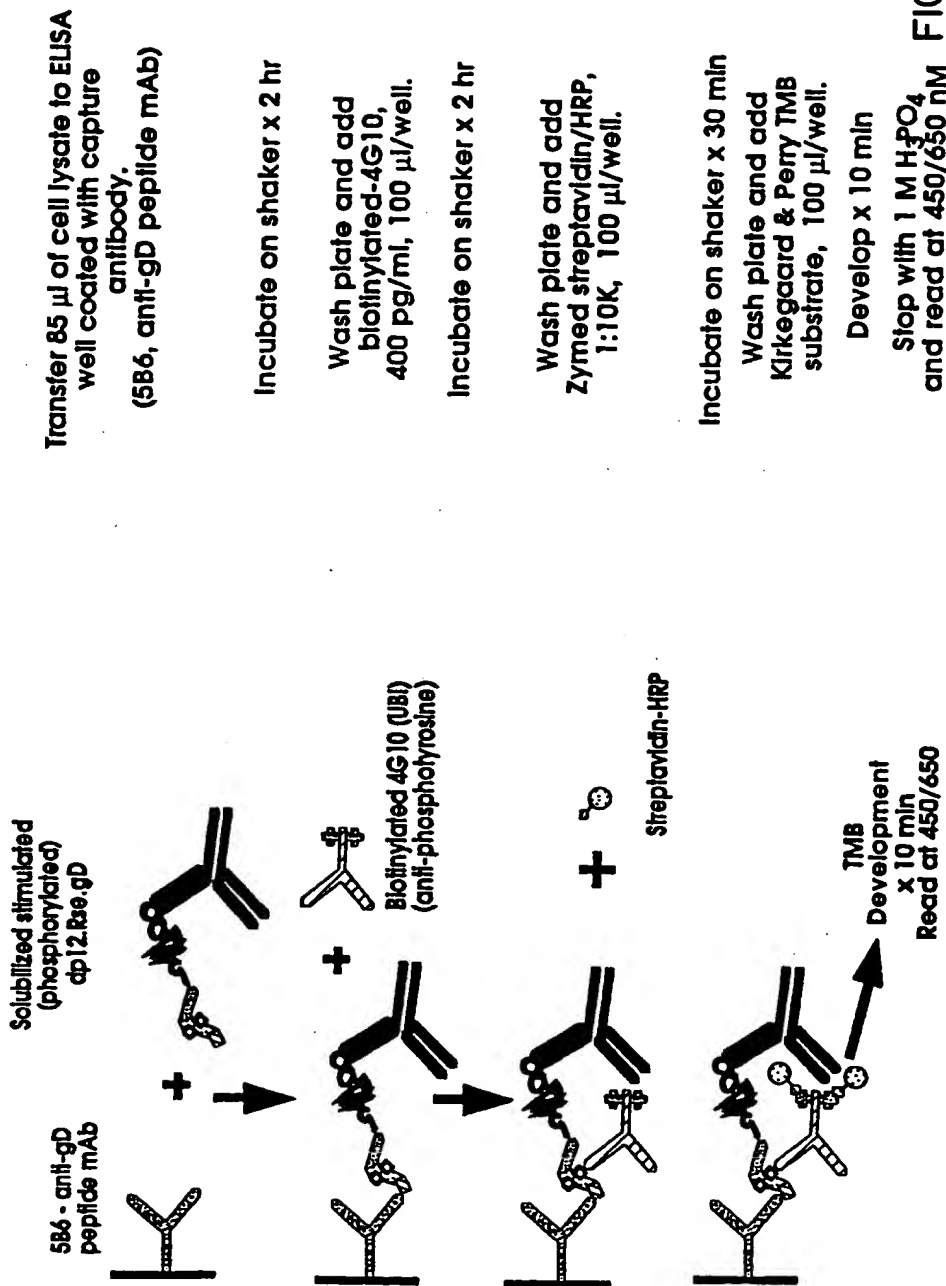


FIG.9B

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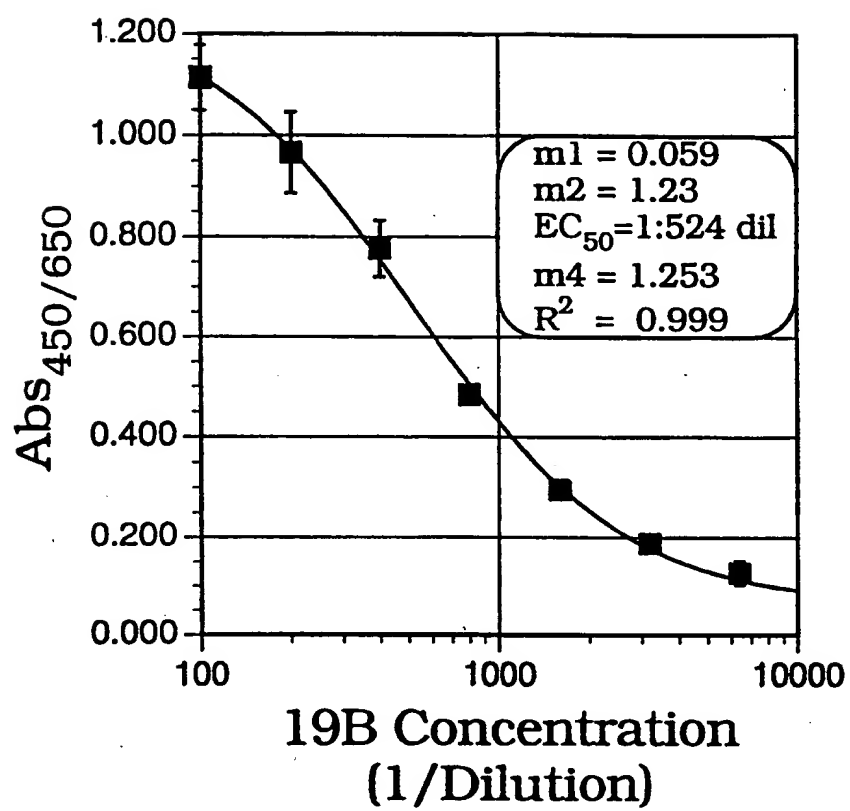
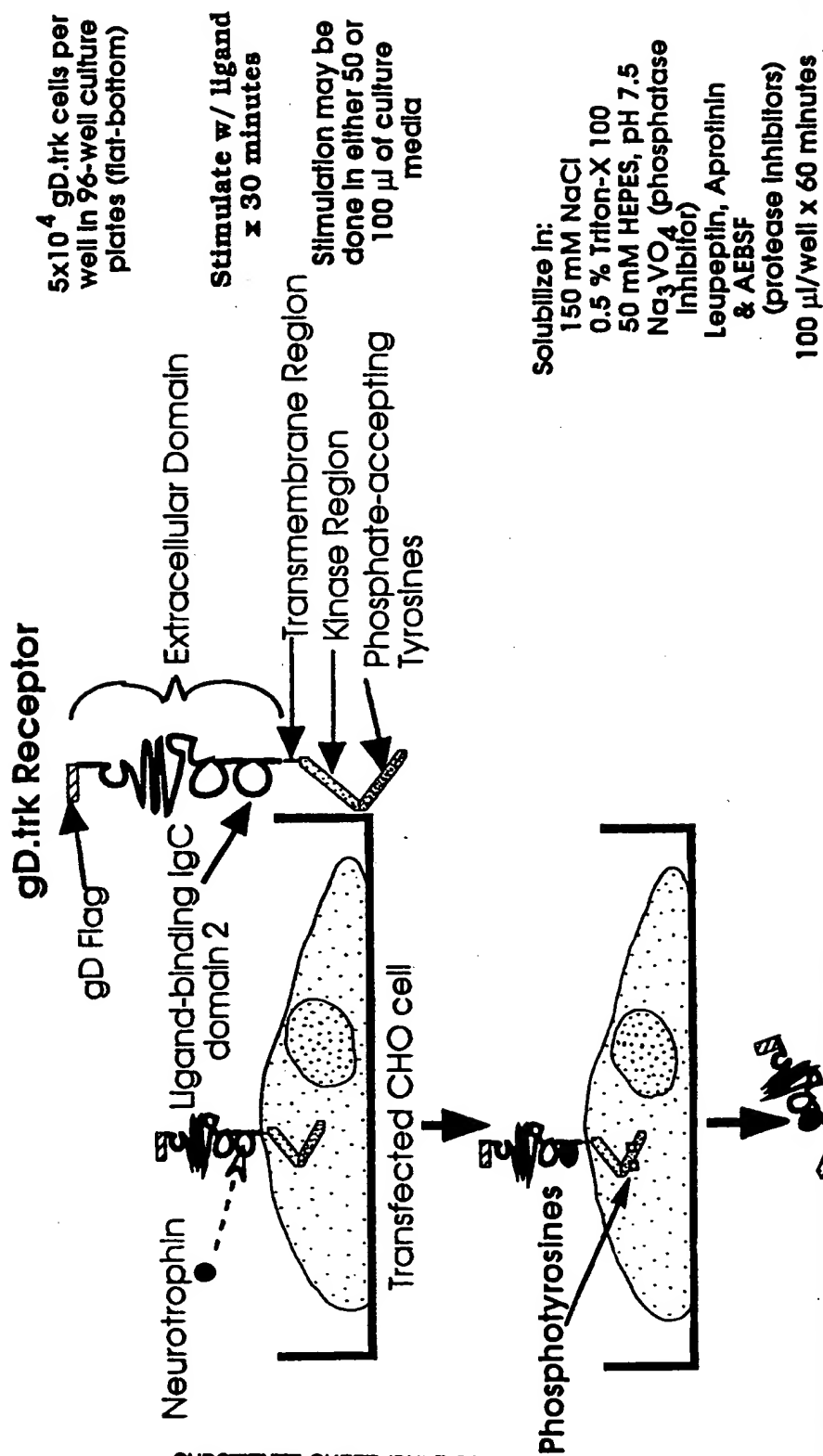


FIG. 10

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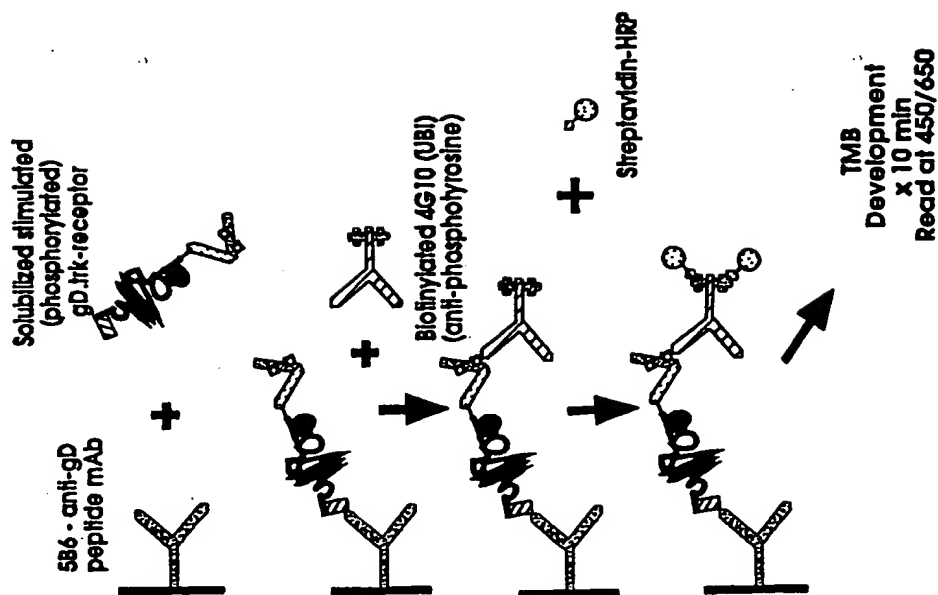


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FIG. 1 | A

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ELISA 96-well plate



Transfer 85 µl of cell lysate to ELISA well coated with capture antibody.
(586, anti-gD peptide mAb)

Incubate on shaker x 2 hr

Wash plate and add biotinylated-4G10, 400 pg/ml, 100 µl/well.

Incubate on shaker x 2 hr

Wash plate and add Zymed streptavidin/HRP, 1:10K, 100 µl/well.

Incubate on shaker x 30 min

Wash plate and add Kirkegaard & Perry TMB substrate, 100 µl/well.

Develop x 10 min

Stop with 1 M H₃PO₄ and read at 450/650 nm

FIG. 1 | B

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```

^sp6 RNA start
841 TATAGAATAA CATCCACTTT GCCTTCTCTT CCACAGGTGT CCACTCCAG GTCCAACGTC
    ATATCTTATT GTAGGTGAAA CGGAAGAGA GGTGTCCACA GGTGAGGGTC CAGGTTGACG

^cloning linker
^R1 site mutated in
^begin gD from pchadII
901 ACCTGAATTC CACTGCCTTC CACCAAGCTC TGCAGGATCC CAGAGTCAGG GGTCTGTATC
    TGGACTTAAG GTGACGGAAG GTGGTTCGAG ACGTCCTAGG GTCTCAGTCC CCAGACATAG

961 TTCCTGCTGG TGGCTCCAGT TCAGGAACAG TAAACCCCTGC TCCGAATATT GCCTCTCACA
    AAGGACGACC ACCGAGGTCA AGTCCTTGTC ATTTGGGACG AGGCTTATAA CGGAGAGTGT

1021 TCTCGTCAAT CTCCGCGAGG ACTGGGGACC CTGTGACAAG CTTCAGCGCG AACGACCAAC
    AGAGCAGTTA GAGGCGCTCC TGACCCCTGG GACACTGTTC GAAGTCGCGC TTGCTGGTTG

1
1081 TACCCCGATC ATCAGTTATC CTTAAGGTCT CTTTGTGTGT GTGCGTTCGG GTATGGGGG
    ATGGGGCTAG TAGTCAATAG GAATTCGAGA GAAACACAC CACGCAAGGC CATAACCCCC

4 T* A* A* R* L* G* A* V* I* L* F* V* V* I* V* G* L* H* G* V*
1141 GACTGCCGCC AGGTTGGGG CCGTGATTTT GTTTGTCTGTC ATAGTGGGCC TCCATGGGGT
    CTGACGGCGG TCCAACCCCC GGCACATAAA CAAACAGCAG TATCACCCCG AGGTACCCCA

24 R* G* K* Y* A* L* A* D* A* S* L* K* M* A* D* P* N* R* F* R
1201 CCGCGGCAAA TATGCCCTTG CCGATGCCTC TCTCAAGATG GCCGACCCCA ATCGATTTCG
    GCGGCCGTTT ATACGGAACC GCCTACGGAG AGAGTTCTAC CGGCTGGGT TAGCTAAAGC

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FIG. 12A

17/70

^Xho and GTA mutated in
 ^begin mature trkA

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44  G K D L P V L D O L L E V A A P C P D A
1261 CGGCAAGAC CTTCCGGTCC TGGACCAGCT GCTCGAGGTA GCCGCACCCCT GCCCCGATGC
    GCCGTTTCTG GAAGGCCAGG ACCTGGTCTGA CGAGTCCAT CGGCGTGGGA CGGGGCTACG

64  C C P H G S S G L R C T R D G A L D S L
1321 CTGCTGCCCC CACGGCTCCT CGGACTGCG ATGCACCCGG GATGGGGCCC TGGATAGCCT
    GACGACGGG GTGCCGAGGA GCCCTGACGC TACGTGGGCC CTACCCCGGG ACCTATCGGA

84  H H L P G A E N L T E L Y I E N Q Q H L
1381 CCACCACCTG CCGGGCGCAG AGAACCCTGAC TGAGCTCTAC ATCGAGAACC AGCAGCATCT
    GGTGGTGGAC GGGCCGCGTC TCTTGGACTG ACTCGAGATG TAGCTCTTGG TCGTCGTAGA

104 Q H L E L R D L R G L G E L R N L T I V
1441 GCAGCATCTG GAGCTCCGTG ATCTGAGGGG CCTGGGGGAG CTGAGAAACC TCACCATCGT
    CGTCGTAGAC CTCGAGGCAC TAGACTCCCC GGACCCCTC GACTCTTGG AGTGGTAGCA

124 K S G L R F V A P D A F H F T P R L S R
1501 GAAGAGTGGT CTCGGTTTCG TGGCGCCAGA TGCCTTCCAT TTCACTCCTC GGCTCAGTCG
    CTTCTCACCA GAGGCAAGC ACCCGGTCT ACGBAAGTA AAGTGAGGAG CCGAGTCAGC

144 L N L S F N A L E S L S W K T V Q G L S
1561 CCTGAATCTC TCCTTCAACG CTCTGGAGTC TCTCTCCTGG AAAACTGTGC AGGCGCTCTC
    GGACTTAGAG AGGAAGTTGC GAGACCTCAG AGAGAGGACC TTTTGACACG TCCCGGAGAG

164 L Q E L V L S G N P L H C S C A L R W L
1621 CTTACAGGAA CTGGTCCTGT CGGGGAACCC TCTGCACTGT TCTTGTGCCC TCGCGTGGCT
    GAATGTCCTT GACCAGGACA GCCCCTTGGG AGACGTGACA AGAACACGGG ACGCGACCGA
  
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FIG. 12B

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184 Q R W E E E G L G G V P E Q K L Q C H G
 1681 ACAGCGCTGG GAGGAGGAGG GACTGGGCGG AGTGCCTGAA CAGAAAGCTGC AGTGTGATGG
 TGTCGCGGACC CTCCTCCTCC CTGACCCGCC CTACGGACTT GTCTTCGACG TCACAGTACC

 204 Q G P L A H M P N A S C G V P T L K V Q
 1741 GCAAGGGCCC CTGGCCACA TGCCCAATGC CAGCTGTGGT GTGCCACGC TGAAGGTCCA
 CGTTCCCGG GACCGGTGT ACGGTTACG GTCGACACCA CACGGGTGG ACTTCCAGGT

 224 V P N A S V D V G D D V L L R C Q V E G
 1801 GGTGCCCCAAT GCCTCGGTGG ATGTGGGGA CGACGTGCTG CTGCGGTGCC AGGTGGAGGG
 CCACGGGTTA CGGAGCCACC TACACCCCT GCTGCACGAC GACGCCACGG TCCACCTCCC

 244 R G L E Q A G W I L T E L E Q S A T V M
 1861 GCGGGCCCTG GAGCAGCCG GCTGGATCCT CACAGAGCTG GAGCAGTCAG CCACGGTGAT
 CGCCCCGGAC CTCGTCCGGC CGACCTAGGA GTGTCTCGAC CTCGTCAATC GGTGCCACTA

 264 K S G G L P S L G L T L A N V T S D L N
 1921 GAAATCTGGG GGTCTGCCAT CCCTGGGGCT GACCTGGCC AATGTCACCA GTGACCTCAA
 CTTTAGACCC CCAGACGGTA GGGACCCCGA CTGGACCCG TTACAGTGGT CACTGGAGTT

 284 R K N L T C W A E N D V G R A E V S V Q
 1981 CAGGAAGAAC TTGACGTGCT GGGCAGAGAA CGATGTGGC CGGGCAGAGG TCTCTGTTCA
 GTCCTTCTTG AACTGCACGA CCCGTCTCTT GCTACACCCG GCCCGTCTCC AGAGACAAAGT

 304 V N V S F P A S V Q L H T A V E M H H W
 2041 GGTCAACGTC TCCTTCCCG CCAGTGTGCA GTGCACACG GCGGTGGAGA TGCACCACTG
 CCAGTTGCAG AGGAAGGGCC GGTCAACAGT CGACGTGTGCG CGCCACCTCT ACGTGGTGAC

FIG. 12C

19/70

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324 C I P F S V D G Q P A P S L R W L F N G
2101 GTGCATCCCC TTCTCTGTGG ATGGCAGCC GGCACGCTCT CTGCGCTGGC TCTTCAATGG
CACGTAGGGG AAGAGACACC TACCCGTGG CCGTGGCAGA GACGCGACCG AGAAGTTACC

344 S V L N E T S F I F T E F L E P A A N E
2161 CTCCGTGCTC AATGAGACCA GCTTCATCTT CACTGAGTTC CTGGAGCCCG CAGCCCAATGA
GAGGCACGAG TTA CTCTGCT CGAAGTAGAA GTGACTCAAG GACCTCGGCC GTCGGTTACT

364 T V R H G C L R L N Q P T H V N N G N Y
2221 GACCGTGCGG CACGGGTGTC TCGCCTCAA CCAGCCCACC CACGTCAACA ACGGCAACTA
CTGGCACGCC GTGCCACAG ACGCGGAGTT GGTGGGTGG GTGCAGTTGT TGCCGTTGAT

384 T L L A A N P F G Q A S A S I M A A F M
2281 CACGCTGCTG GCTGCCAACC CCTCGGCCA GGCTCCGCC TCCATCATGG CTGCCCTTCAT
GTGGACGAC CGACGGTTGG GGAAGCCGGT CCGAGGCGG AGTAGTACC GACGGAAGTA

404 D N P F E F N P E D P I P D T N S T S G
2341 GGACAACCCCT TTCGAGTTCA ACCCGAGGA CCCCATCCCT GACACTAACA GCACATCTGG
CCTGTTGGGA AAGCTCAAGT TGGGCTCCT GGGTAGGGA CTGTGATTGT CGTGTAGACC

^begin TM
424 D P V E K K D E T P F G V S V A V G L A
2401 AGACCCGGTG GAGAAGAAGG ACGAAACACC TTTTGGGGTC TCGGTGGCTG TGGGCCCTGGC
TCTGGGCCAC CTCTTCTTCC TGCTTTGTGG AAAACCCCGAG AGCCACCGAC ACCCGGACCG

^end TM
444 V F A C L F L S T L L L V L N K C G R R
2461 CGTCTTTGCC TGCCTCTTCC TTTCTACGCT GCTCCTGTG CTCAACAAAT GTGGACGGAG
GCAGAAACGG ACGGAGAAGG AAAGATGCCA CGAGGAACAC GAGTTGTTTA CACCTGCCTC

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FIG. 12D

20.70

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464 N K F G I N R P A V L A P E D G L A M S
2521 AAACAAGTTT GGGATCAACC GCCCGGCTGT GCTGGCTCCA GAGGATGGGC TGGCCATGTC
TTTGTTCAAA CCTAGTTGG CGGGCCGACA CGACCGAGGT CTCCTACCCG ACCGGTACAG

484 L H F M T L G G S S L S P T E G K G S G
2581 CCTGCATTTC ATGACATTGG GTGGCAGCTC CCTGTCCCCC ACCGAGGGCA AAGGCTCTGG
GGACGTAAG TACTGTAACC CACCGTCGAG GGACAGGGGG TGGCTCCCGT TTCCGAGACC

504 L Q G H I I E N P Q Y F S D A C V H H I
2641 GCTCCAAGGC CACATCATCG AGAACCCACA ATACTTCAGT GATGCCCTGTG TTCACCCACAT
CGAGGTTCCG GTGTAGTAGC TCTTGGGTGT TATGAAGTCA CTACGGACAC AAGTGGTGTA

524 K R R D I V L K W E L G E G A F G K V F
2701 CAAGCGCCGG GACATCGTGC TCAAGTGGGA GCTGGGGAG GGCGCCTTTG GGAAGGTCTT
GTTTCGGGGCC CTGTAGCACG AGTTCACCCCT CGACCCCCCTC CCGCGGAAAC CCTTCCAGAA

544 L A E C H N L L P E Q D K M L V A V K A
2761 CCTTGCTGAG TGCCACAACC TCCTGCCCTGA GCAGGACAAG ATGCTGGTGG CTGTCAAGGC
GGAACGACTC ACGGTGTTGG AGGACGGACT CGTCCCTGTTT TACGACCACC GACAGTTCCG

564 L K E A S E S A R Q D F Q R E A E L L T
2821 ACTGAAGGAG GCGTCCGAGA GTGCTCGGCA GGACTTCCAA CGTGAGGCTG AGCTGCTCAC
TGACTTCCTC CGCAGGCTCT CACGAGCCGT CCTGAAGGTT GCACTCCGAC TCGACGAGTG

584 M L Q H Q H I V R F F G V C T E G R P L
2881 CATGCTGCAG CACCAGCACA TCGTGGCCTT CTTCGGCGTC TGCACCGAGG GCCGCCCCCT
GTACGACGTC GTGGTCGTGT AGCAGCGGAA GAAGCCGCAG ACGTGGCTCC CGGCGGGGGA

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FIG. 12E

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604 L M V F E Y M R H G D L N R F L R S H G
2941 GCTCATGGTC TTTGAGTATA TCGGCACGG GGACCTCAAC CGTTTCCTCC GATCCCATGG
      CGAGTACCAG AAACATCATAT ACGCCGTGCC CCTGGAGTTG GCGAAGGAGG CTAGGGTACC

624 P D A K L L A G G E D V A P G P L G L G
3001 ACCTGATGCC AAGCTGCTGG CTGGTGGGA GGATGTGGCT CCAGGCCCCC TGGGTCTGGG
      TGGACTACGG TTCGACGACC GACCACCCCT CCTACACCGA GGTCCGGGG ACCCAGACCC

644 Q L L A V A S Q V A A G M V Y L A G L H
3061 GCAGCTGCTG GCCGTGGCTA GCCAGGTGC TGCGGGGATG GTGTACCTGG CGGTCTGCA
      CGTCGACGAC CGGCACCGAT CGGTCCAGCG ACGCCCTAC CACATGGACC GCCAGACGT

664 F V H R D L A T R N C L V G Q G L V V K
3121 TTTTGTGCAC CGGACCTGG CCACACGCA CTGTCTAGTG GGCCAGGGAC TGGTGGTCAA
      AAAACACGTG GCCCTGGACC GGTGTGCGTT GACAGATCAC CCGTCCCTG ACCACCAAGT

684 I G D F G M S R D I Y S T D Y Y R V G G
3181 GATTGGTGAT TTTGGCATGA GCAGGGATAT CTACAGCACC GACTATTACC GTGTGGGAGG
      CTAACCACTA AAACCGTACT CGTCCCTATA GATGTCGTGG CTGATAATGG CACACCCCTC

704 R T M L P I R W M P P E S I L Y R K F T
3241 CCGCACCATG CTGCCCATTC GCTGGATGCC GCCCGAGAGC ATCCTGTACC GTAAGTTCAC
      GCGGTGGTAC GACGGGTAAG CGACCTACGG CCGGCTCTCG TAGGACATGG CATTCAAGTG

724 T E S D V W S F G V V L W E I F T Y G K
3301 CACCGAGAGC GACGTGTGGA GCTTCGGCGT GGTGCTCTGG GAGATCTTCA CCTACGGCAA
      GTGGCTCTCG CTGCACACCT CGAAGCCGCA CCACGAGACC CTCTAGAAGT GGATGCCGTT

744 Q P W Y Q L S N T E A I D C I T Q G R E

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FIG. 12F

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3361 GCAGCCCTGG TACCAGCTCT CCAACACGGA GGAATCGAC TGCATCAGC AGGACGTGA
      CGTCGGACC ATGGTCGAGA GGTGTGCCT CCGTAGCTG ACGTAGTGG TCCCTGCACT

764  L E R P R A C P P E V Y A I M R G C W Q
3421 GTTGGAGCGG CCACGTGCCT GCCCACCAGA GGTCTACGCC ATCATCGGG GCTGCTGGCA
      CAACCTCGCC GGTGCACGGA CGGGTGTCT CCAGATGCGG TAGTACGCCC CGACGACCGT

784  R E P Q Q R H S I K D V H A R L Q A L A
3481 GCGGAGCCC CAGCAACGCC ACAGCATCAA GGATGTGCAC GCCCGGCTGC AAGCCCTGGC
      CGCCCTCGG GTCGTTGCGG TGTCGTAGTT CCTACACGTG CGGGCCGACG TTCGGGACCG

      R1 site added with cloning primer^
      R1 site removed with cut and fill^

804  Q A P P V Y L D V L G O
3541 CCAGGCACCT CCTGTCTACC TGGATGTCCT GGGTAGAAT TAATTCAATC GATGGCCGCC
      GGTCCGTGGA GGACAGATGG ACCTACAGGA CCGATCTTA ATTAAGTTAG CTACCGGCGG

      ^sv40 early poly A

3601 ATGGCCCAAC TTGTTTATTG CAGCTTATAA TGGTTACAAA TAAAGCAATA GCATCACAAA
      TACCGGGTTG AACAAATAAC GTCGAATATT ACCAATGTTT ATTTCGTTAT CGTAGTGTTT

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FIG. 12G

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^sp6 RNA start
841 TATAGAATAA CATCCACTTT GCCTTTCTCT CCACAGGTGT CCACTCCCAG GTCCAACAGTGC
    ATATCTTATT GTAGGTGAA CGGAAGAGA GGTGTCCACA GGTGAGGGTC CAGGTTGACG

^cloning linker ^begin gD from pchadII
901 ACCTCGGTTT TATCGATTGA ATTCACCTGC CTTCACCAA GCTCTGCAGG ATCCACAGAGT
    TGGAGCCAAG ATAGCTAACT TAAGGTGACG GAAGGTGGTT CGAGACGTCC TAGGGTCTCA

961 CAGGGGTCTG TATCTTCCTG CTGGTGGCTC CAGTTCAGGA ACAGTAAACC CTGCTCCGAA
    GTCCCCAGAC ATAGAAGGAC GACCACCGAG GTCAAGTCCT TGTCAATTGG GACGAGGCTT

1021 TATTGCCCTCT CACATCTCGT CAATCTCCGC GAGGACTGGG GACCCCTGTGA CAAGCTTCAG
    ATAACGGAGA GTGTAGAGCA GTTAGAGGCG CTCCTGACCC CTGGGACACT GTTCGAAGTC

1081 CGCGAACGAC CAACTACCCC GATCATCAGT TATCCTTAAG GTCTCTTTTG TGTGGTGCGT
    GCGCTTGCTG GTTGATGGG CTAGTAGTCA ATAGGAATTC CAGAGAAAAC ACACCCACGCA

^Start gD
1 M* G* G* T* A* A* R* L* G* A* V* I* L* F* V* V* I* V*
1141 TCCGGTATGG GGGGACTGC CGCCAGGTG GGGCCGTGA TTTTGTGTTGT CGTCATAGTG
    AGGCCATACC CCCCCTGACG CCGGTCCAAC CCCCAGGCACT AAAACAAACA GCAGTATCAC

19 G* L* H* G* V* R* G* K* Y* A* L* A* D* A* S* L* K* M* A* D
1201 GGCCTCCATG GGTCCGCGG CAAATATGCC TTGGCGGATG CCTCTCTCAA GATGGCCGAC
    CCGGAGGTAC CCCAGGCGC GTTTATACGG AACCGCCTAC GGAGAGAGTT CTACCGGCTG

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FIG. 13A

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xho and GTA mutated in^
start mature trkB^

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39 P N R F R G K D L P V L D O L L E V C P
1261 CCCAATCGAT TTCGGGGCAA AGACCTTCCG GTCCTGGACC AGCTGCTCGA GGTATGTCCC
GGGTTAGCTA AAGCGCCGTT TCTGGAAGGC CAGGACCTGG TCGACGAGCT CCATACAGGG

59 T S C K C S A S R I W C S D P S P G I V
1321 ACGTCCCTGCA AATGCAGTGC CTCTCGGATC TGGTGCAGCG ACCCTTCTCC TGGCATCGTG
TGCAGGACGT TTACGTCACG GAGAGCCTAG ACCACGTCGC TGGGAAGAGG ACCGTAGCAC

79 A F P R L E P N S V D P E N I T E I F I
1381 GCATTTCGGA GATTGGAGCC TAACAGTGTA GATCCTGAGA ACATCACCGA AATTTTCATC
CGTAAAGGCT CTAACCTCGG ATTGTCACAT CTAGGACTCT TGTAGTGGCT TTAAAAGTAG

99 A N Q K R L E I I N E D D V E A Y V G L
1441 GCAAACCCAGA AAAGGTTAGA AATCATCAAC GAAGATGATG TTGAAGCTTA TGTGGGACTG
CGTTTGGTCT TTTCCAATCT TTAGTAGTTG CTTCTACTAC AACTTCGAAT ACACCCGTGAC

119 R N L T I V D S G L K F V A H K A F L K
1501 AGAAATCTGA CAATTGTGGA TTCTGGATTA AAATTTGTGG CTCATAAAGC ATTTCTGAAA
TCITTAGACT GTTAACACCT AAGACCTAAT TTAAACACACC GAGTATTTCCG TAAAGACTTT

139 N S N L Q H I N F T R N K L T S L S R K
1561 AACAGCAACC TGCAGCACAT CAATTTTACC CGAAACAAC TGACGAGTTT GTCTAGGAAA
TTGTCGTTGG ACGTCGTGTA GTTAAATGG GCTTTGTTTG ACTGCTCAAA CAGATCCTTT

159 H F R H L D L S E L I L V G N P F T C S
1621 CATTTCCGTC ACCTTGACTT GTCTGAACTG ATCCTGGTGG GCAATCCATT TACATGCTCC
GTAAAGGCAG TGGAACCTGAA CAGACTTGAC TAGGACCACC CGTTAGGTAA ATGTACGAGG

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FIG. 13B

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179 C D I M W I K T L Q E A K S S P D T Q D
1681 TGTGACATTA TGTGGATCAA GACTCTCCAA GAGGCTAAAT CCAGTCCAGA CACTCAGGAT
      AACTGTGAAT ACACCTAGTT CTGAGAGGTT CTCCGATTTA GGTCCAGGCT GTGAGTCCTA

199 L Y C L N E S S K N I P L A N L Q I P N
1741 TTGTACTGCC TGAATGAAAG CAGCAAGAAT ATTCCCCTGG CAAACCTGCA GATACCCAAT
      AACATGACGG ACTTACTTTC GTCGTTCTTA TAAGGGGACC GTTTGGACGT CTATGGGTTA

219 C G L P S A N L A A P N L T V E E G K S
1801 TGTGGTTTGC CATCTGCAA TCTGGCCGCA CCTAACCTCA CTGTGGAGGA AGGAAAGTCT
      ACACCAAACG GTAGACGTTT AGACCGGCGT GGATTGGAGT GACACCTCCT TCCTTTCAGA

239 I T L S C S V A G D P V P N M Y W D V G
1861 ATCACATTAT CCTGTAGTGT GGCAGGTGAT CCGGTTCCCTA ATATGTATTG GGATGTTGGT
      TAGTGTAATA GGACATCACA CCGTCCACTA GGCCAAGGAT TATACATAAC CCTACAACCA

259 N L V S K H M N E T S H T Q G S L R I T
1921 AACCTGGTTT CCAACATAT GAATGAAACA AGCCACACAC AGGGCTCCTT AAGGATAACT
      TTGGACCAA GGTITGTATA CTTACTTTGT TCGGTGTGTG TCCCGAGGAA TTCCTATTGA

279 N I S S D D S G K Q I S C V A E N L V G
1981 AACATTTTCAT CCGATGACAG TGGGAAGCAG ATCTCTTGTG TGGCGGAAA TCTTGTAGGA
      TTGTAAAGTA GGCTACTGTC ACCCTTCGTC TAGAGAACAC ACCGCCCTTT AGAACATCCT

299 E D Q D S V N L T V H F A P T I T F L E
2041 GAAGATCAAG ATTCTGTCAA CCTCACTGTG CATTTGAC CAACTATCAC ATTTCTCGAA
      CTCTTAGTTC TAAGACAGTT GGAGTGACAC GTAAAACGTG GTTGATAGTG TAAAGAGCTT

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FIG. 13C

26:70

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319 S P T S D H H W C I P F T V K G N P K P
2101 TCTCCAACCT CAGACCACCA CTGGTGCATT CCATTCACTG TGAAGGCAA CCCAAACCA
AGAGGTTGGA GTCTGGTGGT GACCACGTAA GGTAAAGTGAC ACTTCCGTT GGGTTTGGT

339 A L Q W F Y N G A I L N E S K Y I C T K
2161 GCGCTTCAGT GGTTCATATA CGGGGCAATA TTGAATGAGT CCAAATACAT CTGTACTAAA
CGCGAAGTCA CCAAGATATT GCCCCGTTAT AACTTACTCA GGTATTATGA GACATGATTT

359 I H V T N H T E Y H G C L Q L D N P T H
2221 ATACATGTTA CCAATCACAC GGAGTACCAC GGCTGCCCTCC AGCTGGATAA TCCCACTCAC
TATGTACAAT GGTTAGTGTG CCTCATGGTG CCGACGGAGG TCGACCTATT AGGTTGAGTG

379 M N N G D Y T L I A K N E Y G K D E K Q
2281 ATGAACAATG GGGACTACAC TCTAATAGCC AAGAATGAGT ATGGGAAGGA TGAGAAACAG
TACTTGTTAC CCTGATGTG AGATTATCGG TTCTTACTCA TACCCTTCCT ACTCTTTGTC

399 I S A H F M G W P G I D D G A N P N Y P
2341 ATTTCTGCTC ACTTCATGGG CTGGCCTGGA ATTGACGATG GTGCAAAACCC AAATTATCCT
TAAAGACGAG TGAAGTACCC GACCGGACCT TAACTGCTAC CACGTTTGGG TTTAATAGGA

419 D V I Y E D Y G T A A N D I G D T T N R
2401 GATGTAATTT ATGAAGATTA TGAACCTGCA GCGAATGACA TCGGGGACAC CACGAACAGA
CTACATTAAA TACTTCTAAT ACCTTGACGT CGCTTACTGT AGCCCCCTGT GTGCTTGTCT

439 S N E I P S T D V T D K T G R E H L S V
2461 AGTAATGAAA TCCCTTCCAC AGACGTCAC TATAAAACCG GTCGGGAACA TCTCTCGGTC
TCATTACTTT AGGGAAGGTG TCTGCAGTGA CTATTTTGGC CAGCCCCTGT AGAGAGCCAG

459 Y A V V V I A S V V G F C L L V M L F L
2521 TATGCTGTGG TGGTGATTGC GTCTGTGGTG GGATTTTGCC TTTTGGTAAT GCTGTTTCTG
ATACGACACC ACCACTAACG CAGACACCAC CCTAAAACGG AAAACCATTA CGACAAAGAC

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FIG. 13D

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479 L K L A R H S K F G M K G P A S V I S N
2581 CTTAAGTTGG CAAGACACTC CAAGTTTGGC ATGAAAGGCC CAGCCTCCGT TATCAGCAAT
    GAATTCACCC GTTCTGTGAG GTTCAAACCG TACTTTCGG GTCCGAGGCA ATAGTCGTTA

499 D D S A S P L H H I S N G S N T P S S
2641 GATGATGACT CTGCCAGCCC ACTCCATCAC ATCTCCAATG GGAGTAACAC TCCATCTTCT
    CTACTACTGA GACGGTCCGG TGAGGTAGTG TAGAGGTTAC CCTCATTTGT AGGTAGAAGA

519 S E G G P D A V I I G M T K I P V I E N
2701 TCGGAAGGTG GCCCAGATGC TGTCAATTATT GGAATGACCA AGATCCCCTGT CATTGAAAAAT
    AGCCTTCCAC CGGGTCTACG ACAGTAATAA CCTTACTGGT TCTAGGGACA GTAACCTTTTA

539 P Q Y F G I T N S Q L K P D T F V Q H I
2761 CCCCAGTACT TTGGCATCAC CAACAGTCAG CTCAGGCCAG ACACATTTGT TCAGCACATC
    GGGGTCATGA AACCGTAGTG GTTGTCAAGTC GAGTTCGGTC TGTGTAACA AGTCGTGTAG

559 K R H N I V L K R E L G E G A F G K V F
2821 AAGCGACATA ACATTGTTCT GAAAAGGGAG CTAGGCGAAG GAGCCTTTGG AAAAGTGTTC
    TTCGCTGTAT TGTAAACAAGA CTTTTCCTC GATCCGCTTC CTCGGAAACC TTTTCACAAG

579 L A E C Y N L C P E Q D K I L V A V K T
2881 CTAGCTGAAT GCTATAACCT CTGTCCTGAG CAGGACAAGA TCTTGGTGGC AGTGAAGACC
    GATCGACTTA CGATATTGGA GACAGGACTC GTCCTGTTCT AGAACCACCG TCACTTCTGG

599 L K D A S D N A R K D F H R E A E L L T
2941 CTGAAGGATG CCAGTGACAA TGCACGCAAG GACTTCCACC GTGAGGCCGA GCTCCTGACC
    GACTTCCCTAC GGTCACCTGTT ACGTGCCTTC CTGAAGGTGG CACTCCGGCT CGAGGACTGG

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FIG. 13E

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619 N L Q H E H I V K F Y G V C V E G D P L
3001 AACCTCCAGC ATGAGCACAT CGTCAAGTTC TATGGCGTCT GCGTGGAGGG CGACCCCTTC
TTGGAGGTCG TACTCGTGTA GCAGTTCAAG ATACCGCAGA CGACCTCCC GCTGGGGGAG

639 I M V F E Y M K H G D L N K F L R A H G
3061 ATCATGGTCT TTGAGTACAT GAAGCATGGG GACCTCAACA AGTTCTTCAG GGCACACGGC
TAGTACCAGA AACTCATGTA CTTCGTACCC CTGGAGTTGT TCAAGGAGTC CCGTGTGCCG

659 P D A V L M A E G N P P T E L T Q S Q M
3121 CCTGATGCCG TGCTGATGGC TGAGGGCAAC CCGCCACCG AACTGACGCA GTCGCAGATG
GGACTACGGC ACGACTACCG ACTCCGTTG GCGGGTGCC TTGACTGCGT CAGCGTCTAC

679 L H I A Q Q I A A G M V Y L A S Q H F V
3181 CTGCATATAG CCCAGCAGAT CGCCGCGGC ATGGTCTACC TGGCGTCCCA GCACCTCGTG
GACGTATATC GGGTCGTCTA GCGGCGCCG TACCAGATGG ACCGACGGT CGTGAAGCAC

699 H R D L A T R N C L V G E N L L V K I G
3241 CACCGCGATT TGGCCACCAG GAACTGCCCTG GTCGGGGAGA ACTTGCTGGT GAAAATCGGG
GTGGCGCTAA ACCGGTGTC CTTGACGGAC CAGCCCTCT TGAACGACCA CTTTTAGCCC

719 D F G M S R D V Y S T D Y Y R V G G H T
3301 GACTTTGGGA TGTCCCGGGA CGGTACAGC ACTGACTACT ACAGGGTCGG TGGCCACACA
CTGAAACCCCT ACAGGGCCCT GCACATGTCG TGAATGATGA TGTCCCAGCC ACCGGTGTGT

739 M L P I R W M P P E S I M Y R K F T T E
3361 ATGCTGCCCA TTCGCTGGAT GCCTCCAGAG AGCATCATGT ACAGGAAATT CACGACGGAA
TACGACGGGT AAGCGACCTA CGGAGGTCTC TCGTAGTACA TGTCTTTTAA GTGCTGCCCTT

759 S D V W S L G V V L W E I F T Y G K Q P
3421 AGCGACGTCT GGAGCCTGGG GGTCGTGTTG TGGGAGATTT TCACCTATGG CAAACAGCCC

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FIG. 13F

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TCGCTGCAGA CCTCGGACCC CCAGCACAA ACCCTCTAAA AGTGGATACC GTTTGTCCGG

779 W Y Q L S N N E V I E C I T Q G R V L Q
 3481 TGGTACCAGC TGTCAAACAA TGAGGTGATA GAGTGTATCA CTCAGGGCCG AGTCCTGCAG
 ACCATGGTCG ACAGTTTGTT ACTCCACTAT CTCACATAGT GAGTCCCGGC TCAGGACGTC

799 R P R T C P Q E V Y E L M L G C W Q R E
 3541 CGACCCCGCA CGTGCCCCCA GGAGGTGTAT GAGCTGATGC TGGGGTGCTG GCAGCGAGAG
 GCTGGGGCGT GCACGGGGGT CCTCCACATA CTCGACTACG ACCCCACGAC CGTCGCTCTC

819 P H M R K N I K G I H T L L Q N L A K A
 3601 CCCACATGA GGAAGAACAT CAAGGGCATC CATACCCTCC TTCAGAACTT GGCCAAAGGCA
 GGGGTGTA CTCTCTTGTA GTTCCCGTAG GTATGGGAGG AAGTCTTGAA CCGGTTCCGT

839 S P V Y L D I L G O
 3661 TCTCCGGTCT ACCTGGACAT TCTAGGCTAG GGCCCTTTTC CCCAGACCGA TCCTTCCCAA
 AGAGGCCAGA TGGACCTGTA AGATCCGATC CCGGAAAAG GGTCTGGCT AGGAAGGGTT

3721 CGTACTCCTC AGACGGGCTG AGAGGATGAA CATCTTTTAA CTGCCGCTGG AGGCCACCAA
 GCATGAGGAG TCTGCCCCGAC TCTCCTACTT GTAGAAAATT GACGGCGACC TCCGGTGGTT

half xho half Sal site from subcloning^

3781 GCTGCTCTCC TTCACTCTGA CAGTATTAA ATCAAGACT CCGAGAAGCT CTCGACCTGC
 CGACGAGAGG AAGTGAGACT GTCATAATTG TAGTTTCTGA GGCTCTTCGA GAGCTGGACG

^sv40 early poly A

3841 AGAAGCTTGG CCGCCATGSC CCAACTTGTT TATTGCAGCT TATAATGGTT ACAAATAAAG
 TCTTCGAACC GCGGGTACCG GGTGGAACAA ATAACGTGCA ATATTACCAA TGTTTATTTC

FIG. 13G

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```

^sp6 RNA start
841 TATAGATAAA CATCCACTTT GCCTTTCTCT CCACAGGTGT CCACTCCCAG GTCCAACTGC
    ATATCTTATT GTAGGTGAAA CGGAAGAGA GGTGTCCACA GGTGAGGGTC CAGGTTGACG

^cloning linker
^RI site mutated in
^gd from pchadII
901 ACCTGAATTC CACTGCCTTC CACCAAGCTC TGCAGGATCC CAGAGTCAGG GGTCTGTATC
    TGGACTTAAG GTGACGGAAG GTGGTTCGAG ACGTCCTAGG GTCTCAGTCC CCAGACATAG

961 TTCCCTGCTGG TGGCTCCAGT TCAGGAACAG TAAACCCCTGC TCCGAATATT GCCTCTCACA
    AAGGACGACC ACCGAGGTCA AGTCCTTGTC ATTTGGGACG AGGCTTATAA CGGAGAGTGT

1021 TCTCGTCAAT CTCCGCGAGG ACTGGGGACC CTGTGACAAG CTTCAGCGCG AACGACCAAC
    AGAGCAGTTA GAGGCGCTCC TGACCCCTGG GACACTGTTC GAAGTCGCGC TTGCTGGTTG

^Start
          M* G* G*
          1 TACCCCGATC ATCAGTTATC CTTAAGGTCT CTTTGTGTG GTGCGTTCCG GTATGGGGGG
          ATGGGGCTAG TAGTCAATAG GAATTCGAGA GAAACACAC CACGCAAGGC CATACCCCCC

          4 T* A* A* R* L* G* A* V* I* L* F* V* V* I* V* G* L* H* G* V*
          1141 GACTGCCGCC AGGTTGGGG CCGTGATTT GTTTGTGTC ATAGTGGGC TCCATGGGGT
              CTGACGGCGG TCCAACCCCC GGCACATAAA CAAACAGCAG TATCACCCCG AGGTACCCCA

          24 R* G* K* Y* A* L* A D A S L K M A D P N R F R
          1201 CCGCGGCAAA TATGCCCTGG CGGATGCCCTC TCTCAAGATG GCCGACCCCA ATCGATTTCG
              GCGCCGTTT ATACGGAACC GCCTACGGAG AGAGTTCTAC CGGCTGGGGT TAGCTAAAGC

```

FIG. 14A

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^Xho site and GTA mutated in
 ^begin mature trkC
 C P A N C V C
 L E V
 D O L
 L P V L D O L
 G K D L P V L D O L
 44 1261 CGGCAAGAC CTTCGGGTCC TGGACCAGCT GCTCAGGTA TGCCTGCAA ATTGTGTCTG
 GCCGTTTCTG GAAGGCCAGG ACCTGGTCGA CGAGCTCCAT ACGGACGTT TAACACAGAC
 64 1321 CAGCAAGACT GAGATCAATT GCCGGCGGCC GGACGATGG AACCTCTTCC CCCTCCTGGA
 GTCGTTCTGA CTCTAGTTAA CGGCCGCCGG CCTGCTACCC TTGGAGAAGG GGGAGGACCT
 84 1381 AGGCAGGAT TCAGGGAACA GCAATGGGAA CGCCAATATC AACATCACGG ACATCTCAAG
 TCCCGTCCCTA AGTCCCTTGT CGTTACCCCTT GCGGTTATAG TTGTAGTGCC TGTAGAGTTC
 104 1441 GAATATCACT TCCATACACA TAGAGAACTG GCGCAGTCTT CACACGCTCA ACGCCGTGGA
 CTTATAGTGA AGGTATGTGT ATCTCTTGAC CGCGTCAGAA GTGTGCGAGT TCGGGCACCT
 124 1501 CATGGAGCTC TACACCGGAC TTCAAAGCT GACCATCAAG AACTCAGGAC TTCGGAGCAT
 GTACCTCGAG ATGTGGCCTG AAGTTTTCGA CTGGTAGTTC TTGAGTCCCTG AAGCCTCGTA
 144 1561 TCAGCCCCAGA GCCTTTGCCA AGAACCCCA TTTGCGTTAT ATAAACCTGT CAAGTAACCG
 AGTCGGGTCT CGGAAACGGT TCTTGGGGGT AAACGCAATA TATTGGACA GTTCATTTGC
 164 1621 GCTCACCACA CTCTCGTGGC AGCTCTTCCA GACGCTGAGT CTTCCGGGAAT TGCAGTTGGA
 L T T L S W Q L F Q T L S L R E L Q L E
 CTTCCGGGAAT TGCAGTTGGA

FIG. 14B

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CGAGTGGTGT GAGAGCACCG TCGAGAAGGT CTGCGACTCA GAAGCCCTTA ACGTCAACCT
 184 Q N F F N C S C D I R W M Q L W Q E Q G
 1681 GCAGAACTTT TTCAACTGCA GCTGTGACAT CCGCTGGATG CAGCTCTGGC AGGAGCAGGG
 CGTCTTGAAA AAGTTGACGT CGACACTGTA GCGGACCTAC GTCGAGACCG TCCTCGTCCC
 204 E A K L N S Q N L Y C I N A D G S Q L P
 1741 GGAGGCCAAG CTCACACGCC AGAACCTCTA CTGCATCAAT GCTGATGGCT CCCAGCTTCC
 CCTCCGGTTC GAGTTGTCCG TCTTGGAGAT GACGTAGTTA CGACTACCGA GGTTCGAAGG
 224 L F R M N I S Q C D L P E I S V S H V N
 1801 TCTCTTCCGC ATGAACATCA GTCAGTGTGA CCTTCCCTGAG ATCAGCGTGA GCCACGTCAA
 AGAGAAGGCG TACTTGTAGT CAGTCACACT GGAAGGACTC TAGTCGCACT CCGTGCAGTT
 244 L T V R E G D N A V I T C N G S G S P L
 1861 CCTGACCGTA CGAGAGGGTG ACAATGCTGT TATCACTTGC AATGGCTCTG GATCACCCCT
 GGACTGGCAT GCTCTCCAC TGTTACGACA ATAGTGAACG TTACCGAGAC CTAGTGGGA
 264 P D V D W I V T G L Q S I N T H Q T N L
 1921 TCCTGATGTG GACTGGATAG TCACTGGGCT GCAGTCCATC AACACTCACC AGACCAATCT
 AGGACTACAC CTGACCTATC AGTGACCCGA CGTCAGGTAG TTGTGAGTGG TCTGGTTAGA
 284 N W T N V H A I N L T L V N V T S E D N
 1981 GAACTGGACC AATGTTTCATG CCATCAACTT GACGCTGGTG AATGTGACGA GTGAGGACAA
 CTTGACCTGG TTACAAGTAC GGTAGTTGAA CTGCGACCCAC TTACACTGCT CACTCCCTGTT
 304 G F T L T C I A E N V V G M S N A S V A
 2041 TGGCTTCACC CTGACGTGCA TTGCAGAGAA CGTGGTGGGC ATGAGCAATG CCAGTGTTC
 ACCGAAGTGG GACTGCACGT AACGTCTCTT GCACCACCCG TACTCGTTAC GGTCAACAACG

FIG. 14C

SUBSTITUTE SHEET (RULE 26)

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```

324 L T V Y Y P P R V V S L E E P E L R L E
2101 CCTCACTGTC TACTATCCCC CACGTGTGGT GAGCCTGAGC TGCCTGGA
GGAGTGACAG ATGATAGGGG GTGCACACCA CTCGGACCTC CTCGGACTCG ACGCGGACCT

344 H C I E F V V R G N P P P T L H W L H N
2161 GCACTGCATC GAGTTGTGG TCGTGGCAA CCCCCACCA ACGCTGCACT GGCTGCACAA
CGTGACGTAG CTCAAACACC ACGCACCGTT GGGGGTGGT TCGACGTGA CCGACGTGTT

364 G Q P L R E S K I I H V E Y Y Q E G E I
2221 TGGGCAGCCT CTGCGGAGT CCAAGATCAT CCATGTGGAA TACTACCAAG AGGAGAGAT
ACCGTCCGA GACGCCCTCA GGTCTTAGTA GTACACCTT ATGATGGTTC TCCCTCTCTA

384 S E G C L L F N K P T H Y N N G N Y T L
2281 TTCCGAGGC TGCCTGCTCT TCAACAAGCC CACCCACTAC AACAAATGGCA ACTATACCT
AAGCTCCCC ACGGACGAGA AGTTGTTCCG GTGGGTGATG TTGTTACCGT TGATATGGGA

404 I A K N P L G T A N Q T I N G H F L K E
2341 CATGCCAAA AACCCACTGG GCACAGCCAA CCAGACCATC AATGGCCACT TCCTCAAGGA
GTAACGGTTT TTGGGTGACC CGTGTGCGTT GTCTGGTAG TTACCGGTGA AGGAGTTCCT

^begin ecd insert ^end ecd insert
424 P F P E S T D N F I L F D E V S P T P P
2401 GCCCTTTCCA GAGAGCAGG ATAACCTTAT CTTGTTTAC GAAGTGAGTC CCACACCTCC
CGGAAAGGT CTCTCGTGCC TATTGAAATA GAACAACTG CTTCACTCAG GGTGTGGAGG

^begin TM
444 I T V T H K P E E D T F G V S I A V G L
2461 TATCACTGTG ACCCACAAC CAGAAGAAGA CACTTTTGGG GTATCCATAG CAGTTGGACT

```

FIG. 14D

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ATAGTGACAC TGGGTGTTTG GTCTTCTTCT GTGAAAACCC CATAGGTATC GTCAACCTGA

^end TM

464 A A F A C V L L V V L F V M I N K Y G R
2521 TGCTGCTTTT GCCTGTGTC TGTTGGTGGT TCTCTTCGTC ATGATCAACA AATATGGTCG
ACGACGAAA CGGACACAGG ACAACCACCA AGAGAAGCAG TACTAGTTGT TTATACCAGC

484 R S K F G M K G P V A V I S G E E D S A
2581 ACGGTCCAAA TTTGGAATGA AGGTCCCGT GGCTGTCAATC AGTGGTGAGG AGGACTCAGC
TGCCAGGTTT AAACCTTACT TCCCAGGGCA CCGACAGTAG TCACCACCTCC TCCTGAGTCG

504 S P L H H I N H G I T T P S S L D A G P
2641 CAGCCCACTG CACCACATCA ACCACGGCAT CACCACGCCC TCGTCACTGG ATGCCCCGGC
GTCGGGTGAC GTGGTGTAGT TGGTGCCGTA GTGGTCCGG AGCAGTGACC TACGGCCCCG

524 D T V V I G M T R I P V I E N P Q Y F R
2701 CGACACTGTG GTCATTGGCA TGACTCGCAT CCCTGTCAAT GAGAACCCCC AGTACTTCCG
GCTGTGACAC CAGTAACCGT ACTGAGCGTA GGGACAGTAA CTCTTGGGG TCATGAAGGC

544 Q G H N C H K P D T Y V Q H I K R R D I
2761 TCAGGGACAC AACTGCCACA AGCCGGACAC GTATGTGCAG CACATTAAGA GGAGAGACAT
AGTCCCTGTG TTGACGGTGT TCGGCCCTGT CATAACGTC GTGTAATTCT CCTCTCTGTA

^begin TK

564 V L K R E L G E G A F G K V F L A E C Y
2821 CGTGCTGAAG CGAGAACTGG GTGAGGGAGC CTTTGGAAG GTCTTCCCTGG CCGAGTGCTA
GCACGACTTC GCTCTTGACC CACTCCCTCG GAAACCTTC CAGAAGGACC GGCTCAGCAT

FIG. 14E

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```

584 N L S P T K D K M L V A V K A L K D P T
2881 CAACCTCAGC CCGACCAAGG ACAAGATGCT TGTGGCTGTG AAGGCCCTGA AGGATCCCAC
    GTTGGAGTCG GGCTGGTTCC TGTTCTACGA ACACCGACAC TTCCGGGACT TCCTAGGGTG

604 L A A R K D F Q R E A E L L T N L Q H E
2941 CCTGGCTGCC CGGAAGGATT TCCAGAGGGA GGCCGAGCTG CTCACCAACC TGCAGCATGA
    GGACCGACGG GCCTTCCTAA AGGTCTCCCT CCGGCTCGAC GAGTGGTTGG ACGTCGTACT

624 H I V K F Y G V C G D G D P L I M V F E
3001 GCACATTGTC AAGTTCTATG GAGTGTCCGG CGATGGGGAC CCCCTCATCA TGGTCTTTGA
    CGTGTAACAG TTCAAGATAC CTCACACGCC GCTACCCCTG GGGGAGTAGT ACCAGAACT

644 Y M K H G D L N K F L R A H G P D A M I
3061 ATACATGAAG CATGGAGACC TGAATAAGTT CCTCAGGGCC CATGGGCCAG ATGCAATGAT
    TATGTACTTC GTACCTCTGG ACTTATTCAA GGAGTCCCGG GTACCCGGTC TACGTTACTA

664 L V D G Q P R Q A K G E L G L S Q M L H
3121 CCTTGTGGAT GGACAGCCAC GCCAGGCCAA GGGTGAGCTG GGGCTCTCCC AAATGCTCCA
    GGAACACCTA CCTGTCCGGT CCGTCCGGTT CCCACTCGAC CCCGAGAGGG TTTACGAGGT

684 I A S Q I A S G M V Y L A S Q H F V H R
3181 CATTGCCAGT CAGATCGCCT CGGGTATGGT GTACCTGGCC TCCAGCACT TTGTGCACCG
    GTAACGGTCA GTCTAGCGGA GCCCATACCA CATGGACCGG AGGTCGTGA AACACGTGGC

704 D L A T R N C L V G A N L L V K I G D F
3241 AGACCTGGCC ACCAGGAACT GCCTGGTTGG AGCGAATCTG CTAGTGAAGA TTGGGGACTT
    TCTGGACCGG TGGTCCTTGA CGGACCAACC TCGCTTAGAC GATCACTTCT AACCCCTGAA

724 G M S R D V Y S T D Y Y R V G G H T M L
    ^TK insert site

```

FIG. 14F

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```

3301 CGGCATGTCC AGAGATGTCT ACAGCACGGA TTATTACAGG GTGGGAGGAC ACACCATGCT
      GCCGTACAGG TCTCTACAGA TGTCGTGCCT AATAATGTCC CACCCTCCTG TGTGGTACGA

744  P I R W M P P E S I M Y R K F T T E S D
3361 CCCCATTCGC TGGATGCCTC CTGAAAGCAT CATGTACCGG AAGTTCAC TA CAGAGAGTGA
      GGGGTAAGCG ACCTACGGAG GACTTTCGTA GTACATGGCC TTCAAGTGAT GTCTCTCACT

764  V W S F G V I L W E I F T Y G K Q P W F
3421 TGATGGAGC TTCGGGGTGA TCCTCTGGGA GATCTTCACC TATGGAAGC AGCCATGGTT
      ACATACCTCG AAGCCCCACT AGGAGACCCT CTAGAAGTGG ATACCTTTCG TCGGTACCAA

784  Q L S N T E V I E C I T Q G R V L E R P
3481 CCAACTCTCA AACACGGAGG TCATTGAGTG CATTACCCAA GGTCGTGTTT TGGAGCGGCC
      GGTGAGAGT TTGTGCCCTCC AGTAACTCAC GTAATGGGTT CCAGCACAAA ACCTCGCCCG

804  R V C P K E V Y D V M L G C W Q R E P Q
3541 CCGAGTCTGC CCCAAAGAGG TGTACGATGT CATGCTGGGG TGCTGGCAGA GGGAACCACA
      GGCTCAGACG GGGTTTCTCC ACATGCTACA GTACGACCCC ACGACCGTCT CCCTTGGTGT

824  Q R L N I K E I Y K I L H A L G K A T P
3601 GCAGCGGTG AACATCAAGG AGATCTACAA AATCCTCCAT GCTTTGGGGA AGGCCACCCC
      CGTCGCCAAC TTGTAGTTCC TCTAGATGTT TTAGGAGGTA CGAAACCCCT TCCGGTGGGG

      ^stop
      R1 site removed with cut and fill^

844  I Y L D I L G O
3661 AATCTACCTG GACATTCTTG GCTAGTGGTG GCTGGTGGTC ATGAATTAAT TCAATCGATG
      TTAGATGGAC CTGTAAGAAC CGATCACCAC CGACCACCAG TACTTAATTA AGTTAGCTAC

      ^sv40 early poly A
3721 GCCGCCATGG CCCAACTTGT TTATTGCAGC TTATAATGGT TACAAATAAA GCAATAGCAT
      CGCGGTACC GGGTTGAACA AATAACGTCG AATATTACCA ATGTTATTTT CGTTATCGTA

```

FIG. 14G

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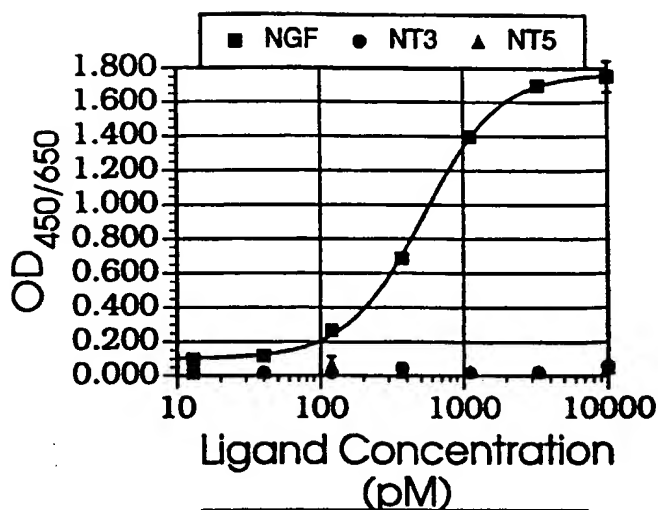
gD.TrkA

FIG. 15A

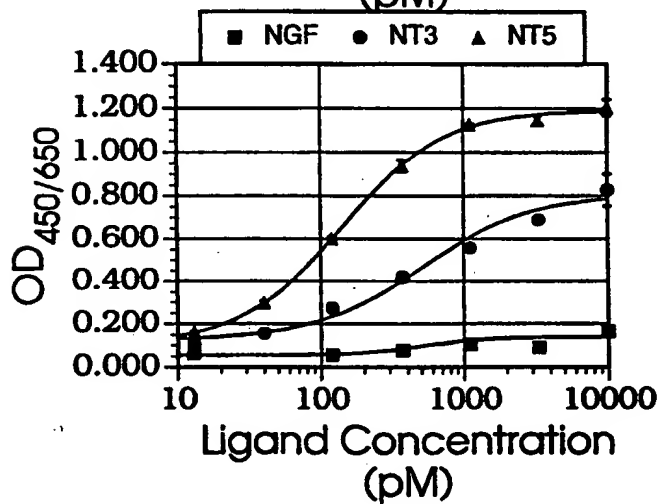
gD.TrkB

FIG. 15B

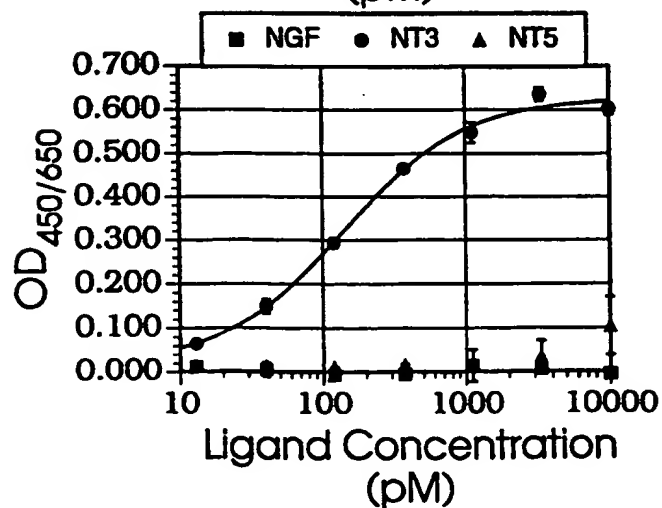
gD.TrkC

FIG. 15C

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```

aluI      aluI
sstI      sau3AI pvuII
sacI      mboI/ndeII[dam-]
hgiJII    dpnI[dam+]
hgiAI/aspHI  pvuI/bspCI
ec1136II  pleI dpnII[dam-]
bsp1286   hinfI taqI[dam-]
bsiHKAI   rmaI mcrI nspBII
bmyI      maeI taqI[dam-]
banII     taqI
1 TTCGAGCTCG CCGACATTG ATTATTGACT AGAGTCGATC GACAGCTGTG GAATGTGTGT CAGTTAGGGT
AAGCTCGAGC GGGCTGTAAC TAATAACTGA TCTCAGCTAG CTGTCGACAC CTTACACACA GTCAATCCCA

nlaIV
scrFI     sfaNI
mvaI      ppulOI
ecorII    nsII/avaIII
dsav      nlaIII
bstNI     sphI
apyI[dcm+] nspI
bsaJI     nspHI
71 GTGGAAGTC CCCAGGCTCC CCAGCAGGCA GAAGTATGCA AAGCATGCAT CTCAATTAGT CAGCAACCAG
CACCTTTCAG GGTCCGAGG GGTCCGCTCCG CTTCATACGT TTCGTACGTA GAGTTAATCA GTCGTTGGTC

```

FIG. 16A

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```

nlaIV          sfaNI          ppulOI
scrFI          mvaI          nsII/avaIII
ecoRII         dsav          nlaIII
                bstNI         sphI
                apyI[dcM+]    nspI
                bsaJI         nspHI
141 GTGTGGAAG TCCCCAGGCT CCCCAGCAGG CAGAAGTATG CAAAGCATGC ATCTCAATTA GTCAGCAACC
    CACACCTTC AGGGTCCGA GGGGTCGTCC GTCTTCATAC GTTTCGTACG TAGAGTTAAT CAGTCGTTGG
                [dcM+]
                acII          fnu4HI
                acII foki    bglI
211 ATAGTCCCGC CCCTAACTCC GCCCATCCCG CCCCTAACTC CGCCCAAGTC CGCCCAATTCT CCGCCCCCATG
    TATCAGGGCG GGGATTGAG GGGTAGGGC GGGGATTGAG GCGGGTCAAG GCGGGTAAGA GCGGGGGTAC
                acII          bslI dsal
                acII bsrI acII acII bsaJI
                fnu4HI         nlaIII
                bglI          styI
                sfiI          ncoI
                haeIII/pali   bslI dsal
                mnlI mnlI     acII bsaJI
                haeIII/pali   acII bsaJI
                mnlI bsaJI    acII bsaJI
                haeIII/pali   acII bsaJI
281 GCTGACTAAT TTTTTTTATT TATGCAGAGG CCGAGGCCGC CTCGGCCTCT GAGCTATTCC AGAAGTAGTG
    CGACTGATTA AAAAAAATAA ATACGTCTCC GGCTCCGGCG GAGCCGGAGA CTCGATAAGG TCTTCATCAC
                mnlI

```

FIG. 16B

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```

rmaI          haeIII/palI
styI          mcrI
bsaJI         eagI/xmaIII/ecI XI
blnI          eaeI
avrII         cfri
haeIII/palI  mspi
stuI          nhei
haeI          mspI
mnII maeI    hpaII
mnII maeI    aluI
351 AGGAGGCTTT TTTGGAGGCC TAGGCTTTTG CAAAAGCTA GCTTATCCGG
TCCTCCGAAA AACCTCCGG ATCCGAAAC GTTTTCGAT CGAATAGGCC

          tfII
scrFI        hinFI
ncII         aciI
mspi         thal
hpaII        fnuDII/mvni
dsav         bstUI
cauII        bsh1236I
401 CCGGGAACGG TGCATTGGAA CGCGGATTCC CCGTGCCAAG AGTGACGTAA GTACCGCCTA TAGAGCGATA
GGCCCTTGCC ACGTAACCTT GCGCCTAAGG GGCACGGTTC TCACTGCATT CATGGCGGAT ATCTCGCTAT
          maeII rsal csp6I scfI
          maeIII
          ^splice donor

          fnu4HI
          bbvI
          nspBII
          aciI
          nlaIII taqI
          sfaNI
          pflMI
          bslI
          471 AGAGGATTTT ATCCCGCTG CCATCATGGT TCGACCATG AACTGCATCG TCGCCGTGTC CCAAAATATG
TCTCCTAAAA TAGGGCGCAC GGTAATACCA AGCTGGTAAC TTGACGTAGC AGCGGCACAG GGTTTTATAC
          DHFR ATG^

```

FIG. 16C

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```

haeIII/palI
haeI
scrFI
mvaI      bsrBI
ecorII
dsav
bstNI     aciI      rsal      csp6I
          apyI[dcM+]  xmnI     asp700  scaI
          bsaJI     mnII     ddeI
541 GGGATTGGCA AGAACGGAGA CCTACCTGG CCTCCGCTCA GGAACGAGTT CAAGTACTTC CAAAGAATGA
    CCTAACCGT TCTTGCCTCT GGATGGGACC GGAGCGGAGT CCTTGCTCAA GTTCATGAAG GTTCTTACT

          bsmAI
          bsal
          bstNI     aciI      rsal      csp6I
          apyI[dcM+]  xmnI     asp700  scaI
          bsaJI     mnII     ddeI
611 CCACAACCTC TTCAGTGGAA GGTAAACAGA ATCTGGTGAT TATGGGTAGG AAAACCTGGT TCTCCATTCC
    GGTGTTGGAG AAGTCACCTT CCATTGTCT TAGACCACTA ATACCCATCC TTTTGGACCA AGAGGTAAGG

          eco57I
          mboII
          earI/ksp632I
          mnII
          tfiI
          hinfI
          alwNI     hphI
          tru9I     msei
          mboII     taqI     ahaIII/draI     asei/asnI/vspi
          mboII taqI CGACCTTTAA AGGACAGAAT TAATATAGTT CTCAGTAGAG AACTCAAAGA ACCACCACGA
          ACTCTTCTTA GCTGGAAATT TCCTGTCTTA ATTATATCAA GAGTCATCTC TTGAGTTTCT TGGTGTGCT

          bslI     mnII

```

FIG. 16D

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```

sstI
sacI
hgiJII
hgiAI/aspHI
ec1136II
bsp1286
bsiHKAI
bmyI
banII
aluI
bstXI
fokI
sfanI
mseI
aflIII/bfrI
tru9I
mspI
hpaII
bsaWI
751 GGAGCTCATT TTCCTGCCAA AAGTTTGGAT GATGCCCTTAA GACTTATTGA ACAACCGGAA TTGGCAAAGTA
CCTCGAGTAA AAGAACGGTT TTCAAACCTA CTACCGAATT CTGAATAACT TGTGGCCTT AACCGTTCAT

accI
nlaIII
mnlI
821 AAGTAGACAT GGTTCGGATA GTCGGAGGCA GTTCTGTTTA CCAGGAAGCC ATGAATCAAC CAGGCCACCT
TTCATCTGTA CCAAACCTAT CAGCCTCCGT CAAGACAAAT GGTCTTCGG TACTTAGTTG GTCCGGTGGA

scrFI scrFI scrFI
mvaI mvaI mvaI
ecorII ecorII ecorII
dsaV dsaV dsaV
bstNI bstNI bstNI
nlaIII nlaIII nlaIII
hinfI hinfI hinfI
apyI[dcM+] apyI[dcM+] apyI[dcM+]
dclI dclI dclI
haeIII/palI
haeI

```

FIG. 16E

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nlaIII
 sau3AI
 mboI/ndeII(dam-)
 dpnI(dam+)
 dpnII(dam-)
 pleI
 hinfI
 maeIII alwI(dam-) apoI
 maeII
 aflIII
 maeIII
 891 TAGACTCTTT GTGACAAAGGA TCATGCAGGA ATTTGAAAGT GACACGTTT TCCCAGAAAT TGATTGGGG
 ATCTGAGAAA CACTGTTCT AGTACGTCCT TAAACTTCA CTGTGCAAAA AGGTCTTTA ACTAAACCCC

hgaI
 hinfI/acyI
 ahaII/bsaHI
 scrFI
 mvaI
 ecorII
 dsav
 bstNI
 apyI(dcm+) mnlI
 bsaJI
 bsli ddeI
 mnlI
 961 AAATATAAAC CTCTCCCAGA ATACCCAGGC GTCTCTCTG
 TTTATATTG GAGAGGGTCT TATGGGTCCG CAGGAGAGAC

FIG. 16F

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```

scrFI
mvaI
ecorII
dsaV
bstNI
apyI[dcn+]
sau96I
avaII
asuI      mnlI      sfanI      accI      mboII      mboII      sfanI
1001 AGGTCAGGA GGAAGAGGC ATCAAGTATA AGTTTGAAGT CTACGAGAAG AAAGACTAAC AGGAAGATGC
TCCAGGTCCT CCTTTTCCG TAGTTCATAT TCAAACTTCA GATGCTCTTC TTTCTGATTG TCCTTCTACG
^END DHER

nlaIII
styI
ncoI
dsal
ppulOI
mnlI      aluI      nsII/avaIII      bsaJI
1071 TTTCAAGTC TCTGCTCCCC TCCTAAAGCT ATGCATTTT ATAAGACCAT GGGACTTTTG
AAAGTTCAAG AGACGAGGGG AGGATTTTCA TACGTAAAAA TATCTGGTA CCCTGAAAC

```

FIG. 16G

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```

          styI
          bsaJI
          sau3AI
          mboI/ndeII[dam-]
          dpnI[dam+]
          dpnII[dam-]
          alwI[dam-]
          bstYI/xhoII
          fnu4HI
          aciI
          thaI
          fnuDII/mvnI tru9I
          bstUI mseI
          bsh1236I aseI/asnI/vspI
          CTGCGTTTAG ATCCCCTTGG CTTCGTTAGA ACGCGGCTAC AATTATATACA TAACCTTATG TATCATACAC
          GACCGAAATC TAGGGAACC GAAGCAATCT TCGCCGATG TTAATTATGT ATTGGAATAC ATAGTATGTG
          maeIII
          hphI scfI foki
          ATACGATTTA GGTGACACTA TAGATAACAT CCACTTTGCC TTTCTCTCCA CAGGTGTCCA CTCCCAGGTC
          TATGCTAAAT CCACTGTGAT ATCTATTGTA GTGAAACGG AAAGAGAGGT GTCCACAGGT GAGGGTCCAG
          sau96I
          avaiI
          asuI
          scrFI
          mvaI
          ecorII
          dsav
          bstNI
          apyI[dcn+]
          bslI bsaJI

```

FIG. 16H

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```

scrFI
ncII
mspI
hpaII
dsav
xmaI/pspAI
smaI
scrFI
ncII
dsav
cauII
bsaJI
avaI
sau3AI
mboI/ndeII[dam-]
dpsI[dam+]
dpsII[dam-]
pleI
nlaIV cauII
bstYI/xhoII
taqI rmaI bamHI bsaJI
hinfi
sali maeI alwI[dam-]
scfI
alul pstI
hindIII bspMI hincII/hindII alwI[dam-]
accI xbaI mnlI bsaJI
mnlI
bsaJI
1271 CAACTGCACC TCGGTTCTAA GCTTCTGCAG GTCGACTCTA GAGGATCCCC
      GTTGACGTGG AGCCAAGATT CGAAGACGTC CAGCTGAGAT CTCCTAGGGG

```

FIG. 16I

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```

sau96I      acII haeIII/palI
              fnu4HI asuI
              bgII nlaIII
              sfII styI
              eaeI ncoI
              cfrI dsal
              taqI haeIII/palI
              clai/bsp106 bsajI
              ecorI      GGGGAATTCA ATCGATGGCC GCCATGGCCC AACTTGTTTA TTGAACAAAT AACGTCGAAT ATTACCAATG TTTATTTCGT
              apol      CCCCTTAAGT TAGCTACCGG CGGTACCGGG ^sv40
              maeIII
              aluI
              fnu4HI
              bbvI
              maeIII
              rmaI
              bsmI maeI
              sfaNI      apol      ATAGCATCAC AAATTTTACA AATAAAGCAT TTTTTCAC TTTTTCAC TGTGGTTGT CCAAACTCAT
              TATCGTAGTG TTAAAGTG TATTTCGTA AAAAAGTGA CGTAAGATCA ACACCAACA GGTTCAGTA

```

FIG. 16J

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```

sau3AI
mboI/ndeII[dam-]
dpnI[dam+]
dpnII[dam-]
pvuI/bspCI
mcrI
  taqI[dam-] tru9I
    claiI/bsp106[dam-]
sau3AI mseI
mboI/ndeII[dam-]
dpnI[dam+] xmnI
dpnII[dam-] aseI/asnI/vspi
nlaIII alwI[dam-] asp700
1461 CAATGTATCT TATCATGTCT GGATCGATCG GGAATTAAAT
    GTTACATAGA ATAGTACAGA CCTAGCTAGC CCTTAATTAA
      sv40 origin^

nlaIII
haeIII/palI
haeI
  styI
    fnu4HI ncoI
      bbvI dsal
        hinPI bsaJI
          hhaI/cfoI nlaIII
1501 CGGCGCAGCA CCATGGCCTG AAATAACCTC TGAAGAGGA ACTTGGTTAG GTACCTTCTG AGGCGGAAAG
    GCCGCTCGT GTACCCGGAC TTTATTGGAG ACTTCTCCT TGAACCAATC CATGGAAGAC TCCGCCTTTC

```

FIG. 16K

FIG. 16L

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```

                                nlaIII
                                styI
                                ncoI
                                bsII dsal
                                acII bsaJI
                                acII bsaJI
                                TCCGCCCAT GGCTGACTAA TTTTITAT TTATGCAGAG
1781 CCGCCAGTT CCGCCATTC TCCGCCCAT GGCTGACTAA TTTTITAT TTATGCAGAG
                                CCGACTGATT AAAAAATA AATACGTCTC
                                GCGGGTCAA GCGGGTAAG AGCGGGGTA CCGACTGATT AAAAAATA AATACGTCTC

                                fnu4HI
                                bglI
                                sfiI
                                haeIII/palI
                                mnlI mnlI ddeI
                                haeIII/palI bsaJI mnlI aluI
                                bsaJI acII haeIII/palI
1841 GCCGAGGCCG CCTCGGCCTC TGAGCTATTC CAGAAGTAGT GAGGAGGCTT TTTGGAGGC
                                CGGCTCCGGC GGAGCCGGAG ACTCGATAAG GTCTTCATCA CTCCTCCGAA AAAACCTCCG
                                styI
                                bsaJI
                                blnI
                                avrII
                                haeIII/palI
                                stuI
                                haeI
                                mnlI
                                mnlI
                                mnlI
                                GAGGAGGCTT TTTGGAGGC
                                CTCCTCCGAA AAAACCTCCG

```

FIG. 16M

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hinPI
 thai
 fnuDII/mvni
 bstUI scfI
 bsh1236I
 rsal hhai/cfoI fnu4HI
 csp6I bsII acII
 2171 TGTGCGGTAT TTCACACCGC ATACGTCAA GCAACCATAG TACGCGCCCT GTAGCGGCGC
 ACACGCCATA AAGTGTGCG TATGCAGTTT CGTTGSTATC ATGCGCGGGA CATCGCCGCG
 acII acII maeII
 fnu4HI
 thai
 fnuDII/mvni
 bstUI
 hinPI acII
 hhai/cfoI
 tru9I acII
 mseI bsh1236I
 2231 ATTAAGCGCG GCGGGTGTGG TGGTTACGCG CAGCGTGACC GCTACACTTG CCAGCGCCCT AGCGCCCGCT
 TAATTGCGCG CGCCACACACC ACCAATGCGC GTCGCACTGG CGATGTGAAC GGTCGCGGGA TCGCGGCGGA
 fnu4HI
 hinPI
 hhai/cfoI
 thai
 fnuDII/mvni
 bstUI
 bsh1236I
 maeIII bbvI maeIII
 acII
 hinPI haeII
 hhai/cfoI bsrBI
 haeII maeI acII
 2301 CCTTTCGCTT TCTTCCCTTC CTTTCTCGCC ACGTTGCGCG GCTTCCCGG TCAAGCTCTA AATCGGGGCG
 GGAAAGCGAA AGAAGGGAAG GAAAGAGCGG TGCAAGCGGC CGAAGGGGC AGTTCGAGAT TTAGCCCCCG
 mboII
 maeII cfr10I
 aluI
 mspl
 hpaII
 naeI
 nlaIV
 hgiJII
 bsp1286
 bmyI
 banII

FIG. 1 6P

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```

                mnli
                nlaIV
                hgiCI
                bani   taqI
                hphI
2371  TCCCTTTAGG GTTCCGATT AGTGCTTTAC GGCACCTCGA CCCCCAAAAA CTTGATTGG
      AGGGAATCC CAAGGCTAAA TCACGAAATG CCGTGGAGCT GGGTTTTTT GAACATAACC

                nlaIV
                maeII   haeIII/palI
                draIII   sau96I
                bsaAI   asuI
2401  GTGATGGTTC ACGTAGTGGG CCATCGCCCT GATAGACGGT TTTTCGCCCT TTGACGTTGG AGTCCACGTT
      CACTACCAAG TGCATCACCC GGTAGCGGGA CTATCTGCCA AAAAGCGGGA AACTGCAACC TCAGGTGCAA

                tru9I   pleI
                msei   hinfi
                bslI   bsrI
2501  CTTTAATAGT GGACTCTTGT TCCAACTGG AACAACTC AACCCATCT CGGGCTATTC TTTTGATTTA
      GAAATTATCA CCTGAGAACA AGTTTGACC TTGTTGTGAG TTGGGATAGA GCCCGATAAG AAAACTAAAT

                tru9I   msei
                haeIII/palI   msei
                aluI   apoI
2571  TAAGGGATT TGCCGATTTC GGCCTATTGG TTAATAAATG AGCTGATTTA ACAAAAATTT
      ATTCCCTAAA ACGGCTAAAG CCGGATAACC AATTTTAC TCGACTAAAT TGTTTTTAAA

```

FIG. 16Q

SUBSTITUTE SHEET (RULE 26)

[illegible]

FIG. 16T


```

3441 ATGGCATGAC AGTAAGAGAA TTATGCA GTG CAGTGAT AACACTGCGG CCAACTTACT
TACCGTACTG TCATTCTCTT AATACGTCAC GACGGTATTG GTACTCACTA TTGTGACGCC GGTGAATGA

nlaIII fnu4HI fnu4HI
nlaIII bbiI nlaIII aciI
haeIII/palI eaeI cfrI fnu4HI
sau96I
sau3AI asuI mboI/ndeII[dam-] dpnII[dam+] dpnII[dam-]
pvuI/bspCI mcrI mnlI aluI aciI nlaIII alwI[dam-]
sau3AI maeIII mboI/ndeII[dam-] dpnII[dam+] dpnII[dam-]
nlaIII
3511 TCTGACAACG ATCGGAGGAC CGAAGAGCT AACCGCTTTT TTGCACAACA TGGGGGATCA TGTAACTCGC
AGACTGTTGC TAGCCTCCTG GCTTCCTCGA TTGGCGAAAA AACGTGTTGT ACCCCCTAGT ACATTGAGCG
mspi
sau3AI nlaIV mboI/ndeII[dam-] aluI dpnII[dam+] hpaII dpnII[dam-] bsaWI
nlaIII fnu4HI
3581 CTTGATCGTT GGAACCGGA GCTGAATGAA GCCATACCAA ACGACGAGCG TGACACCACG ATGCCAGCAG
GAACTAGCAA CCCTTGGCCT CGACTTACTT CGGTATGGTT TGCTGCTCGC ACTGTGGTGC TACGGTCGTC
maeIII sfaNI bbiI fnu4HI

```

FIG. 16V

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```

          hinPI      mspI
          hhaI/cfoI  hpaII
          mstI       scrFI
          avIII/fspI aluI nciI
          maeII      rmaI dsav
          psp1406I   maeI cauII
3651 CAATGGCAAC AACGTTGCGC AAACATATTA ACTTACTCTA GCTTCCCGGC
      GTTACCGTTG TTGCAACGCG TTGATAATT GACCGCTTGA TGAATGAGAT CGAAGGGCCG

          tru9I      foki
          mseI       bsrI
          aseI/asnI/vspi mnlI
3711 AACAAATTAAT AGACTGGATG GAGCGGGATA AAGTTGCAGG ACCACTTCTG CGCTCGGCC TTCCGGCTGG
      TTGTTAATTA TCTGACCTAC CTCCGCCTAT TTCAACGTCC TGGTGAAGAC GCGAGCCGGG AAGGCCGACC

          tru9I      foki
          mseI       bsrI
          aseI/asnI/vspi mnlI
          acII
          sau96I     hpaII
          avall      hinPI asuI mspI
          asuI       hhaI/cfoI hpaII
          bglI
          sau96I     haeIII/palI
          haeIII/palI mspI
          hpaII
          hpaII
          fnuDII/mvnI
          bstUI
          bsmAI acII fnu4HI
          bsaI bsh1236I bbvI
          nlaIV hphI
          gsuI/bpmI
          CTGGAGCCGG TGAGCGTGGG TCTCGCGGTA TCATTGCAGC
          GACCTCGGCC ACTCGCACCC AGAGCGCCAT AGTAACGTCC
3781 CTGGTTTATT GCTGATAAAT
      GACCAATAA CGACTATTTA

```

FIG. 16W

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FIG. 16X

```

sau96I      pleI
asuI        hinfI
nlaIV
bsrI haeIII/palI mnlI      eam1105I
3841 ACTGGGGCCA GATGGTAAGC CCTCCCGTAT CGTAGTTATC TACACGACGG GGAGTCAGGC
TGACCCCGGT CTACCATTCG GGAGGGCATA GCATCAATAG ATGTGCTGCC CCTCAGTCCG

                                     ddeI
                                     sau3AI      nlaIV
                                     mboI/ndeII[dam-]
                                     dpnI[dam+]      hgiCI      tru9I
                                     dpnII[dam-]      banI mnlI      mseI
fokI
3901 AACTATGGAT GAACGAAATA GACAGATCGC TGAGATAGGT GCCTCACTGA TTAAGCATTG
TTGATACCTA CTTGCTTTAT CTGTCTAGCG ACTCTATCCA CGAGTGACT AATTCGTAAC

                                     tru9I
                                     mseI
maeIII      ahaIII/draI      mseI      tru9I
3961 GTAACGTGCA GACCAAGTTT ACTCATATAT ACTTTAGATT GATTTAAAC TTCAATTTTA
CATTGACAGT CTGGTTCAA TGAGTATATA TGAATCTAA CTAATTTTG AAGTAAAAAT

                                     rmaI      sau3AI
                                     sau3AI hphI      mboI/ndeII[dam-]
                                     mboI/ndeII[dam-]
                                     dpnI[dam+]      dpnI[dam+]
                                     dpnII[dam-]      dpnII[dam-]
                                     tru9I bstYI/xhoII      alwI[dam-]
                                     mseI alwI[dam-]      bstYI/xhoII
                                     ahaIII/draI maeI mboII[dam-]
4021 ATTTAAAGG ATCTAGGTGA AGATCCCTTT TGATAATCTC ATGACCAAAA TCCCTTAACG TGAGTTTCG
TAAATTTTC TAGATCCACT TCTAGGAAAA ACTATTAGAG TACTGGTTTT AGGGAATTGC ACTCAAAAGC

```

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FIG. 16Y

```

sau3AI
mboI/ndeII[dam-]
dpnI[dam+] sau3AI
dpnII[dam-] mboI/ndeII[dam-]
bstYI/xhoII dpnI[dam+]
sau3AI alwI[dam-] dpnII[dam-]
mboI/ndeII[dam-] alwI[dam-]
dpnI[dam+] mboII[dam-]
dpnII[dam-] bstYI/xhoII
4091 TTCCACTGAG CGTCAGACCC CGTAGAAAAG ATCAAAGGAT CTTCTTGAGA TCCTTTTITT
AAGGTGACTC GCAGTCTGGG GCATCTTTC TAGTTTCCTA GAAGAACTCT AGGAAAAAAA

thai
fnuDII/mvnI
bstUI
bsh1236I
hinPI fnu4HI
hhaI/cfoI bbvI
4151 CTGCGCGTAA TCTGCTGCTT GCAAAACAAA AAACCACCGC TACCAGCGGT GGTTTGTTTG
GACGCGCATT AGACGACGAA CGTTTGTTTT TTTGGTGGCG ATGGTCGCCA CCAACACAAAC

sau3AI
mboI/ndeII[dam-]
dpnI[dam+]
dpnII[dam-]
alwI[dam-]
mspI
hpaII aluI
4211 CCGGATCAAG AGCTACCAAC TCTTTTCCG AAGTAACTG GCTTCAGCAG AGCGCAGATA CCAAATACTG
GGCCTAGTTC TCGATGGTTG AGAAAAAGGC TTCCATTGAC CGAAGTCGTC TCGCGTCTAT GGTTTATGAC

```

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```

4281 TCCTTCTAGT GTAGCCGTAG TTAGGCCACC ACTTCAAGAA CTCTGTAGCA CCGCCTACAT ACCTCGCTCT
    AGGAAGATCA CATCGGCATC AATCCGGTGG TGAAGTTCTT GAGACATCGT GCGGGAATGA TGGAGCGAGA
    haeIII/palI      bslI      haeI      scfI      aciI      mnlI
    rmaI      maeI
    4281 TCCTTCTAGT GTAGCCGTAG TTAGGCCACC ACTTCAAGAA CTCTGTAGCA CCGCCTACAT ACCTCGCTCT
    AGGAAGATCA CATCGGCATC AATCCGGTGG TGAAGTTCTT GAGACATCGT GCGGGAATGA TGGAGCGAGA

4351 GCTAATCCTG TTACCAAGTG CTGCTGCCAG TGGCGATAAG TCGTGTCTTA CCGGGTTGGA CTCAAGACGA
    CGATTAGGAC AATGGTCACC GACGACGGTC ACCGCTATTC AGCACAGAAT GCGCCAACCT GAGTTCTGCT
    maeIII      bbsI      bbsI      bbsI      pleI      hinfI
    fnu4HI      alwNI      fnu4HI      bbsI      dsav      cauli      hinfI
    4351 GCTAATCCTG TTACCAAGTG CTGCTGCCAG TGGCGATAAG TCGTGTCTTA CCGGGTTGGA CTCAAGACGA
    CGATTAGGAC AATGGTCACC GACGACGGTC ACCGCTATTC AGCACAGAAT GCGCCAACCT GAGTTCTGCT

4421 TAGTTACCGG ATAGGCGCA GCGGTCGGGC TGAACGGGGG GTTCGTGCAC ACAGCCCAGC TTGGAGCGAA
    ATCAATGGCC TATTCCGGCT CGCCAGCCCC ACTTGCCCCC CAAGCACGTG TGTCGGGTGC AACCTCGCTT
    mspI      hpaII      bsaWI      maeIII      nspBII      fnu4HI      bbsI      mcrI      hinPI      aciI      hhaI/cfoI
    4421 TAGTTACCGG ATAGGCGCA GCGGTCGGGC TGAACGGGGG GTTCGTGCAC ACAGCCCAGC TTGGAGCGAA
    ATCAATGGCC TATTCCGGCT CGCCAGCCCC ACTTGCCCCC CAAGCACGTG TGTCGGGTGC AACCTCGCTT

4491 CGACCTACAC CGAACTGAGA TACCTACAGC GTGAGCAATTG AGAAGCGCC ACCTTCCCG AAGGAGAGAA
    GCTGGATGTG GCTTGACTCT ATGGATGTG CACTCGTAAC TCTTTCGGG TCGGAAGGC TTCCCTCTTT
    ddeI      scfI      haeII      hinPI      hhaI/cfoI
    4491 CGACCTACAC CGAACTGAGA TACCTACAGC GTGAGCAATTG AGAAGCGCC ACCTTCCCG AAGGAGAGAA
    GCTGGATGTG GCTTGACTCT ATGGATGTG CACTCGTAAC TCTTTCGGG TCGGAAGGC TTCCCTCTTT

```

FIG. 16Z-1

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```

scrFI      mspI      fnu4HI      hinPI mnlI      bsaJI
mvaI      hpaII      aciI      hhaI/cfoI  aluI apyI[dcm+]
ecorII     bslI      bsaWI      GCGGCAGGGT  CGGAACAGGA  GAGCGCACGA  GGGAGCTTCC  AGGGGGAAC
dsaV      bsNI      TATCCGGTAA  CCGCCTGTCC  ATAGGCCATT  CGCCGTCCCA  GCCTGTGCTC  CTGCGGTGCT  CCCTCGAAGG  TCCCCCTTTG

```

scrFI

```

dsaV      dsav      mnlI drdI  hgaI      taqI      sfaNI
bsNI      bstNI      apyI[dcm+]  GACTTGAGCG  TCGATTTTGG  TGATGCTCGT
4561 GCCTGGTATC  TTTATAGTCC  TGTCGGGTTT  CGCCACCTCT  CGGAGCTTCC  AGGGGGAAC
CGGACCATAG  AAATATCAGG  ACAGCCCCAA  GCGGTGGAGA  CTGAACTCGC  AGCTAAAAAC  ACTACGAGCA

```

```

haeIII/palI
fnu4HI
aciI
thai.bslI
fnuDII/mvni
bstUI
bsh1236I
nlaIV
aciI
4701 CAGGGGGCG  GAGCCTATGG  AAAAACGCCA  GCAACGCGGC
GTCCCCCGCG  CTCGGATACC  TTTTGGCGT  CGTTGCGCCG

```

FIG. 16Z-2

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```

      haeIII/palI
      scrFI
      mvaI bslI
      ecorII
      dsav
      bstNI
      apyI[dcmt]
      nlaIV haeI
      nlaIII
      haeIII/palI nspI
      haeI
      aflIII
      tflI
      hinfI
4741 CTTTTTACGG TTCCTGGCCT TTTGCTGCC TTTTGCTCAC ATGTTCTTTC CTGCGTTATC CCCTGATTCT
      GAAAATGCC AAGGACCGGA AAACGACCGG AAAACGAGTG TACAAGAAAG GACGCAATAG GGGACTAAGA
      fnu4HI
      bsvI
      bsrBI acII
      aluI acII fnu4HI mcrI
4811 GTGGATAACC GTATTACCGC CTTTGAGTGA GCTGATACCG CTCGCCCGCAG CCGAACGACC
      CACCTATTGG CATAATGGCG GAAACTCACT CGACTATGGC GAGCGGCGTC GGCTTGCTGG
      hinPI
      haeII
      sapi hhaI/cfoI
      mboII
      mnII aciI earI/ksp632I
      mnII aciI earI/ksp632I
4871 GAGCGCAGCG AGTCAGTGAG CGAGGAAGCG GAAGAGCGCC CAATACGCAA ACCGCCCTCTC
      CTCGCGTCCG TCAGTCACTC GTCCTTCGC CTTCTCGCGG GTTATGCGTT TGGCGGAGAG

```

FIG. 16Z-3

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```

thai
fnuDII/mvni
bstUI
bsh1236I
hinPI
hhaI/cfoI
thai
fnuDII/mvni
bstUI
tru9I aluI
bsh1236I haeIII/palI pvuII
bslI eaeI tfil aseI/asnI/vspI
aciI cfrI hinfI mseI nspBII
4931 CCCGCGCGTT GCCCGATTCA TTAATCCAGC TGGCAGCACA GGTTTCCCGA CTGGAAAGCG
GGCGCGCAA CCGGCTAAGT AATTAGGTCG ACCGTGCTGT CCAAAGGGCT GACCTTTCGC
bsrI aciI

scrFI
mvaI
ecorII
dsaV
nlaIV bstNI
hgiCI apyI[dcn+]
bani bsajI

tru9I
mseI
maeIII
hinPI
hhaI/cfoI aseI/asnI/vspI mnlI
4991 GGCAGTGAGC GCAACGCAAT TAATGTGAGT TACCTCACTC ATTAGGCACC CCAGGCTTTA CACTTTATGC
CCGTCACCTCG CGTTGCGTTA ATTACACTCA ATGGAGTGAG TAATCCGTGG GGTCCGAAAT GTGAAATACG

```

FIG. 1 6Z-4

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```

mspl      acil      alul      nlaIII
hpaII     bsrBI
5061 TTCCGGCTCG TATGTTGTGT GGAATTGTGA GCGGATAACA ATTCACACA GGAACACAGCT ATGACCATGA
      AAGCCGAGC ATACAACACA CCTTAACACT CGCCTATTGT TAAAGTGTGT CCTTTGTCTGA TACTGGTACT

```

```

tru9I
mseI
aseI/asnI/vspI
xmnI
asp700
5131 TTACGAATTA A
      AATGCTTAAT T

```

FIG. 16Z-5

>length: 5141

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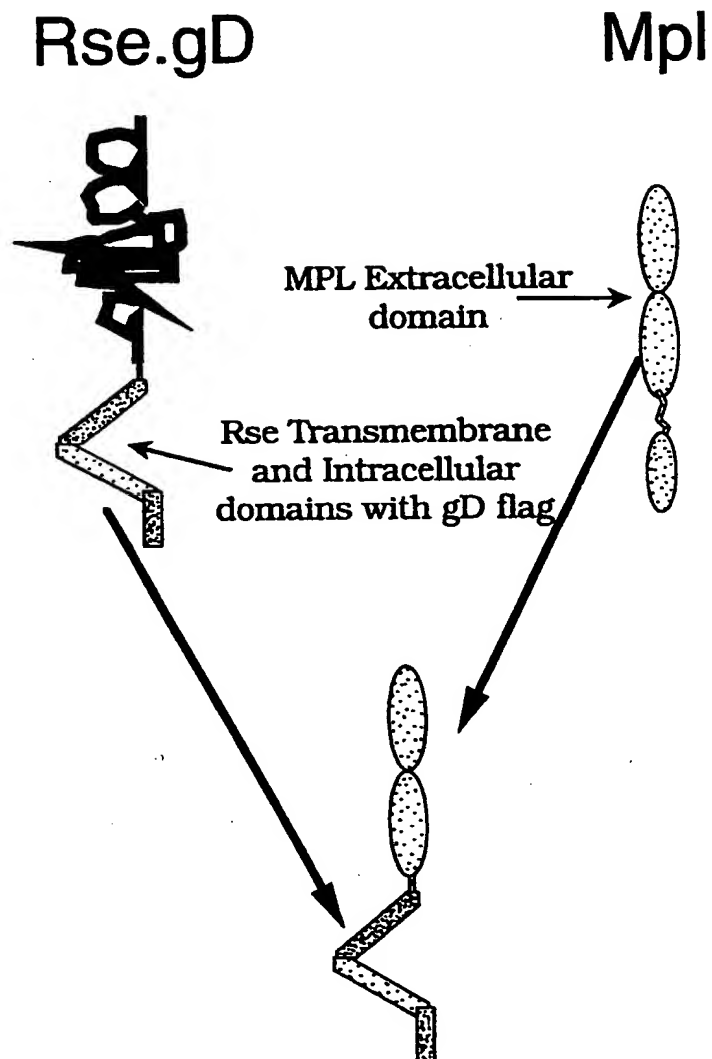


FIG. 17

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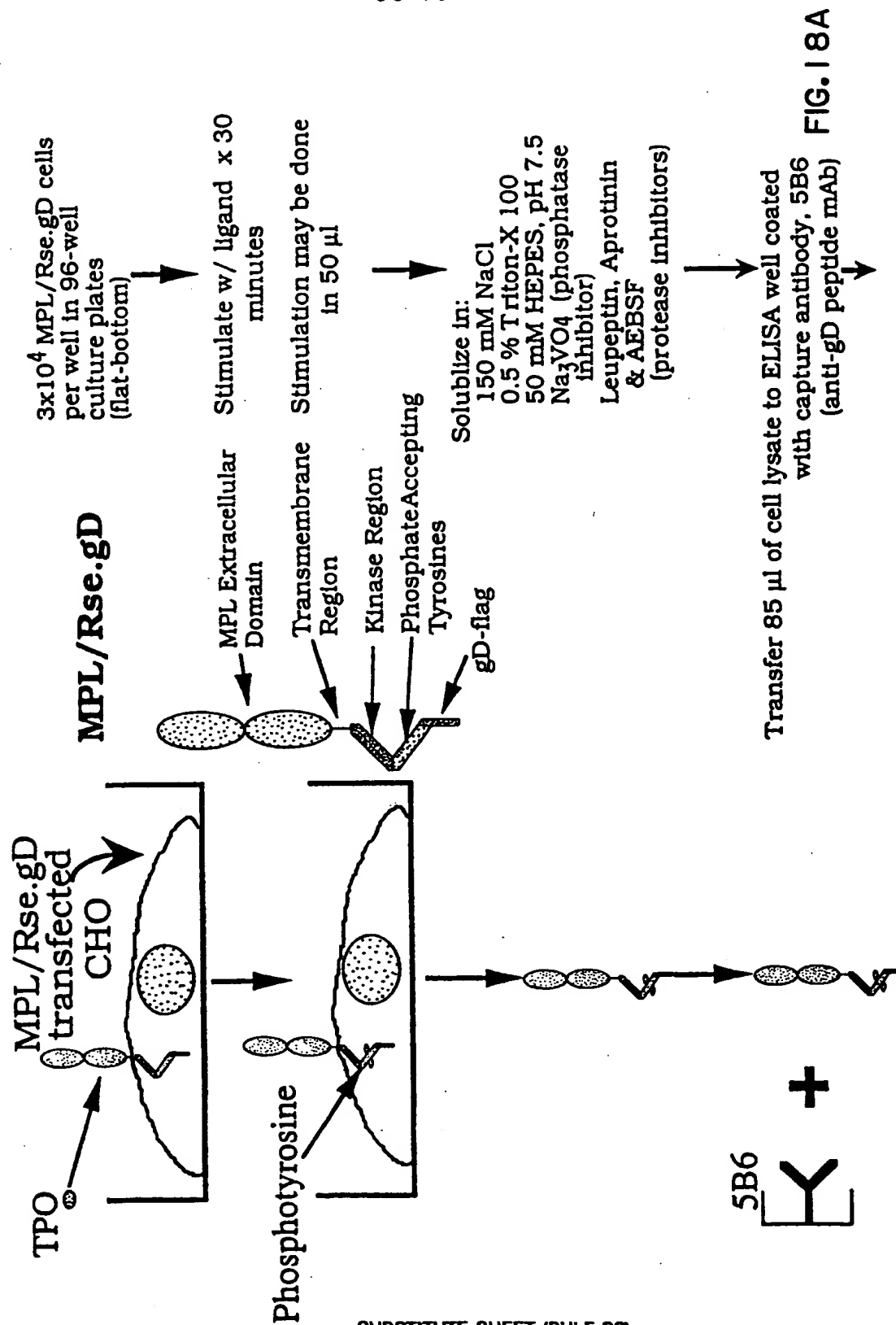


FIG. 18A

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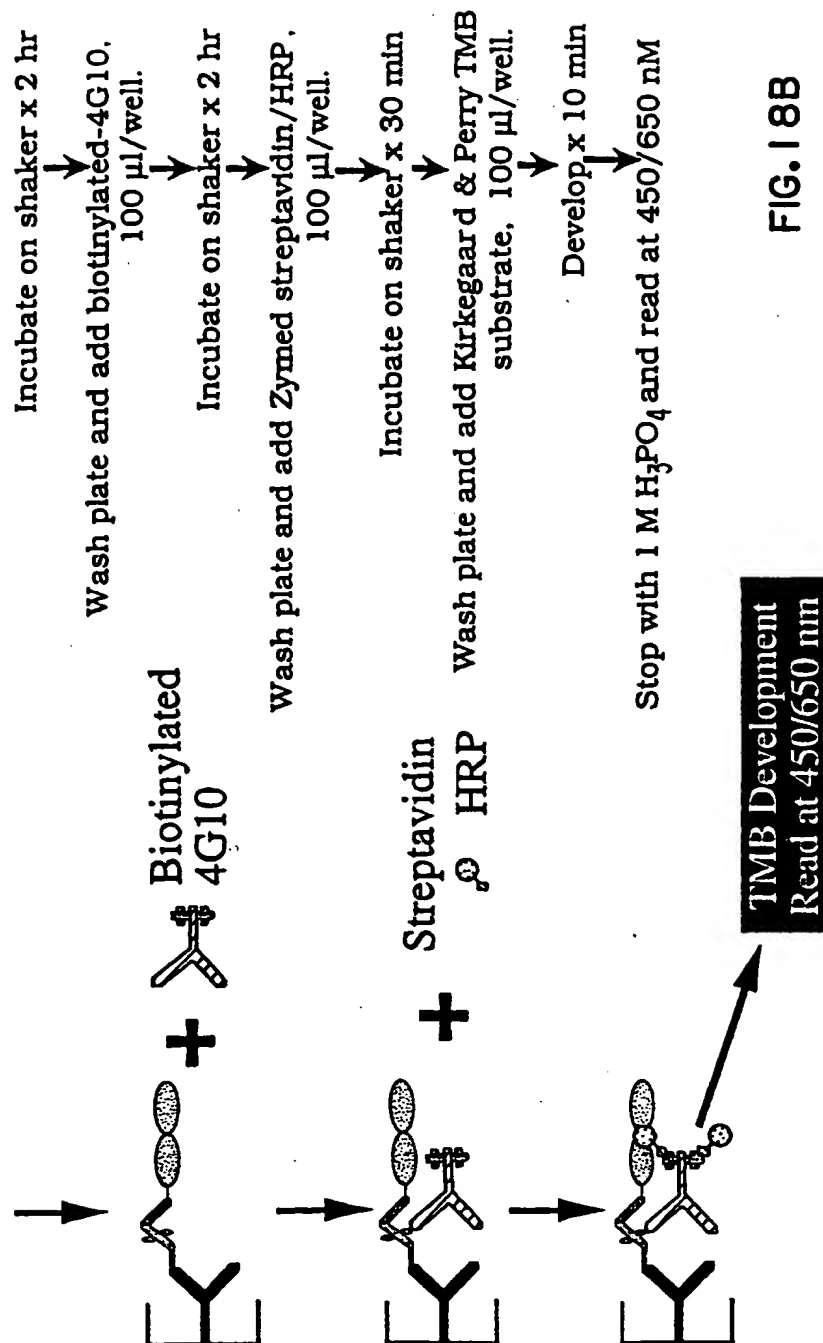


FIG. 18B

INTERNATIONAL SEARCH REPORT

 Internat Application No
 PCT/US 94/13329

 A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 G01N33/573 G01N33/566 G01N33/543 G01N33/532 G01N33/577
 C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

 Minimum documentation searched (classification system followed by classification symbols)
 IPC 6 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	JOURNAL OF BIOLOGICAL CHEMISTRY, vol.269, no.14, 1 April 1994, WASHINGTON DC USA pages 10720 - 10728 M.R. MARK ET AL. 'RSE, a novel receptor-type tyrosine kinase with homology to Axl/Ufo, is expressed at high levels in the brain.' cited in the application	29-32
T	see the whole document see the whole document --- -/--	1-43



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

3 April 1995

Date of mailing of the international search report

27. 04. 95

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 European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
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 Fax (+ 31-70) 340-3016

Authorized officer

Van Bohemen, C

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, vol.285, no.2, 1 March 1991, NEW YORK NY USA pages 197 - 204 V.P. KNUTSON ET AL. 'Comparison of insulin receptor tyrosine phosphorylation under in vitro and in situ conditions: assessment of specific protein tyrosine phosphorylation without the use of 32P-phosphate labeled substrates.' cited in the application see figure 1 ---	1-28, 33-43
Y	DIABETES, vol.42, 1 June 1993, WASHINGTON DC USA pages 883 - 890 H.H. KLEIN ET AL. W 'A microtiter well assay system to measure insulin activation of insulin receptor kinase in intact human mononuclear cells.' cited in the application see page 883, abstract see page 884, column 2, line 9 - line 44 ---	1-28, 33-43
Y	EP,A,0 244 221 (GENENTECH INCORPORATED) 4 November 1987 cited in the application see claim 1 -----	1-28, 33-43

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/13329

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0244221	04-11-87	US-A- 4859609	22-08-89
		CA-A- 1328419	12-04-94
		JP-A- 62272990	27-11-87
		US-A- 5030576	09-07-91
